University of Alberta

Regulation of Receptor Tyrosine Kinases Mediated Signal Transduction by Endocytosis and Phospholipase C-γ1

by

Yi Wang

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Abstract

Receptor Tyrosine Kinases (RTKs) and their signaling pathways have profound effects on various cellular processes. In this thesis, I studied two aspects of RTK signaling: regulation of RTK signaling by endocytosis and the role of phospholipase C-γ1 (PLC-γ1) in RTK-mediated cell signaling.

I first elucidated the poorly understood spatial regulation of RTK signaling. Endocytosis was initially considered as a mechanism to attenuate signaling. Recent evidence has suggested that internalized RTKs may maintain their ability to generate signals from endosomes. However, no research has been able to demonstrate pure endosomal signal and thus prove its physiological relevance. In this thesis, I have successfully established a system to allow the specific activation of endosome-associated RTKs without the initial activation at the plasma membrane. I have provided original evidence demonstrating that endosomes can serve as nucleation sites for the formation of signaling complexes and that endosomal RTK signaling is sufficient to activate major signaling pathways. Endosomal signaling is sufficient to stimulate cell proliferation and anti-apoptotic responses.

The second research area of my thesis is the regulation of RTK-mediated cell signaling by PLC-γ1. Recent results suggest that PLC-γ1 plays a complicated role in RTK-mediated signal transduction and that all PLC-γ1 domains have been implicated in RTK-induced cell signaling. However, the role of PLC-γ1 in various RTK-mediated cell signaling events is still very controversial, and the molecular mechanism by which PLC-

γ1 regulates RTK-mediated cell signaling is far from clear. In this thesis, I have provided original evidence demonstrating that PLC-γ1 translocated to endosomes and remained phosphorylated following its initial translocation from cytosol to the plasma membrane in response to EGF. This translocation is primarily mediated by PLC-γ1's SH2 domains. The PH domain stabilizes PLC-γ1 in the membrane at a later time. I also showed that the SH3 domain of PLC-γ1 mediates its with Akt. This interaction causes the phosphorylation of PLC-γ1 S1248 and plays an important role in EGF-stimulated cell motility.

In summary, the results presented in this thesis provide the basis for a functional understanding of the regulatory mechanisms of RTK signaling.

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List of Abbreviations

AP-1	activator protein 1
ARFs	adenosine diphosphate (ADP)-ribosylation factors
ATP	adenosine triphosphate
BAD	BCL2-antagonist of cell death
BCL2	B-cell CLL/lymphoma 2
BrdU	bromo-deoxy-uridine
Cbl	casitas B-lineage lymphoma
CCV	clathrin-coated vesicle
CR	cysteine-rich
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EEA1	early endosomal autoantigen 1
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
ErbB	erythroblastic leukemia viral oncogene homolog
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FBS	fetal bovine serum
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GH	growth hormone
GHR	growth hormone receptor
Grb2	growth factor receptor bound protein 2
GSK-3	glycogen synthase kinase-3
GTP	guanine triphosphate
GTPases	guanosine triphosphatases
HB-EGF	heparin-binding EGF
IB	immunoblot
IF	immunofluorescence
IgG	immunoglobulin
IP	immunoprecipitation
IRS-1	insulin receptor substrate-1
kD	Kilo Daltons

LR	leucine-rich
MAPK	mitogen-activated protein kinase
MDCK	Madin-Darby Canine Kidney Epithelial
MEK	MAPK kinase
mTOR	mammalian target of rapamycin
NGFR	nerve growth factor receptor
PBS	phosphate buffered saline
PDGFR	platelet-derived growth factor receptor
PDK	3-phosphoinositide dependent protein kinase
PH	pleckstrin homology
PI	phosphoinositide
PI3K	phosphatidyl-inositol 3-kinase
PIKE	PI3K Enhancer
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC-γ1	phospholipase C-γ1
PM	plasma membrane
pS	phsophorylated serine
PTB	phosphotyrosine binding
PtdIns(3,4,5)P_3	phosphatidylinositol 3,4,5-trisphosphate
PtdIns(3)P	phosphatidylinositol 3-phosphate
PtdIns(4,5)P_2	phosphatidylinositol 4,5-bisphosphate
pY	phosphorylated tyrosine
Rac1	ras-related C3 botulinum toxin substrate 1
RBD	Ras binding domain
RhoA	ras homolog gene family, member A
RTKs	receptor tyrosine kinases
SF	serum free
SH2	Src homology 2
SH3	Src homology 3
SHC	Src homologous and collagen like protein
SOS	son-of sevenless
TGF-α	transforming growth factor-alpha
TGF-β	transforming growth factor-beta
TIM	triose phosphate isomerase
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TR-EGF	Texas red-conjugated EGF

Wt	wild type
$InsP_3$	inositol 1, 4, 5-triphosphate
NGF	nerve growth factor
YFP	yellow fluorescent protein

Chapter 1

Introduction

1.1 Overview of Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are central components of cell signaling networks and play crucial roles in normal physiological processes, such as embryogenesis, cell proliferation, cell differentiation, cell metabolism and cell death (apoptosis) as well as cell motility (Reviewed in (Bergeron et al., 1995; Barbieri et al., 2004)). RTK signaling networks function to detect, amplify, filter and process a variety of environmental and intercellular cues (Pawson et al., 2002; Schlessinger, 2000). These networks have been intensively studied for the last 20 years, but only since the completion of the Human Genome Project has the breadth and scope of their regulatory function been apparent (Robinson et al., 2000). For example, a search of the human genome for tyrosine kinase coding elements identified several novel genes and enabled the creation of a non-redundant catalog of tyrosine kinase genes. Fifty-eight unique receptor tyrosine kinase genes can be identified and distributed into 20 subfamilies (Robinson et al., 2000). Unregulated activation of RTKs through mechanisms such as point mutations, over-expression or aberrant cellular localization can lead to various forms of cancer including mammary and squamous carcinomas, glioblastomas, cardiovascular and numerous other malignancies as well as benign proliferative conditions (Bergeron et al., 1995; Barbieri et al., 2004; Pawson et al., 2002; Schlessinger, 2000). The importance of RTKs in health and disease is further underscored by the existence of aberrations in RTK signaling occurring in inflammatory diseases and diabetes (Davies, 1999; Kirvan et al., 2003).

RTKs catalyze transfer of the γ-phosphate of ATP to hydroxyl groups of tyrosine on target proteins (Hunter, 1998). RTKs possess an extracellular ligand binding domain, a transmembrane helix and an intracellular catalytic domain. The extracellular domain is always glycosylated and binds to polypeptide ligands. Characteristically, the extracellular domains are comprised of one or more identifiable structural motifs, including cysteine-rich regions, fibronectin III-like domains, immunoglobulin-like domains, EGF-like domains, cadherin-like domains, kringle-like domains, Factor VIII-like domains, glycine-rich regions, leucine-rich regions, acidic regions and discoidin-like domains. The transmembrane domain anchors the receptor in the plasma membrane, and the cytoplasmic portion of RTKs consists of a juxtamembrane region (just after the transmembrane helix), followed by the tyrosine kinase catalytic domain and a carboxy-teminal regulatory region (Bergeron et al., 1995; Barbieri et al., 2004; Pawson et al., 2002; Schlessinger, 2000). Activation of the kinase is achieved by ligand binding to the extracellular domain, which induces dimerization of the receptors (Lemmon and Schlessinger, 1994). Receptors thus activated are able to autophosphorylate tyrosine residues outside the catalytic domain via cross-phosphorylation. The results of this auto-phosphorylation are stabilization of the active receptor conformation and the creation of phosphotyrosine docking sites for proteins which transduce signals within the cell. Signaling proteins which bind to the intracellular domain of RTKs in a phosphotyrosine-dependent manner always contain SH2 (Src homology region 2), SH3 (Src homology region 3), PH (Pleckstrin homology)

and PTB (phosphotyrosine binding) domains. These proteins include p120 RasGAP, PI3-kinase, phospholipase C, phosphotyrosine phosphatase SHP and adaptor proteins such as SHC, Grb2 and Crk (Bergeron et al., 1995; Barbieri et al., 2004).

1.2 The structure and function of EGFR

The epidermal growth factor (EGF) receptor (EGFR, ErbB1 or HER1) is the first identified RTK and hence one of the most extensively characterized (Carpenter et al., 1978; Carpenter et al., 1979; Hunter and Cooper, 1981; Cohen et al., 1980). *v-ErbB*, identified as one of the oncogenes carried by avian erythroblastosis virus, was shown to encode the homologue of human EGFR, providing one of the first links between oncogenes and human cancers (Frykberg et al., 1983). Since the discovery of EGFR, three additional members of the ErbB family have been identified, designated ErbB2 (HER2/Neu), ErbB3 (HER3) and ErbB4 (HER4) (Ullrich et al., 1984; Coussens et al., 1985; Kraus et al., 1989; Plowman et al., 1993) (Table 1.1). Targeted disruption of the various members of the EGFR family has shown that these receptors play a critical role in the control of a host of cellular activities including cell division, differentiation, and migration. Furthermore, overexpression or dysregulation of EGFR family members has been implicated in a variety of human cancers, such as mammary carcinoma, glioblastomas, and squamous carcinoma (Yarden and Sliwkowski, 2001; Yarden et al., 2001b; Yarden, 2001a; Yarden, 2001b; Yarden et al., 2001a; Frykberg et al., 1983).

Table 1.1 EGFR Family Nomenclature

Nomenclature in Thesis	Other Names in the Literature
EGFR	ErbB-1, HER1
ErbB-2	HER2, neu
ErbB-3	HER3
ErbB-4	HER4

EGFR is a 170 kD transmembrane glycoprotein comprising a single polypeptide chain. Like other RTKs, the receptor consists of an extracellular ligand binding domain, a transmembrane domain, a juxtamembrane region, a tyrosine kinase domain and a carboxy-terminal regulatory domain (Figure 1.1). The heavily glycosylated 622-amino extracellular domain contains two cysteine rich (CR) regions which are responsible for ligand binding. This domain binds at least 6 ligands: EGF, Heparin-binding EGF (HB-EGF), Transforming Growth Factor-alpha (TGF-α), amphiregulin, betacellulin and epiregulin (Toyoda et al., 1995; van der Geer et al., 1994). This domain also mediates the homodimerization of EGFR and the heterodimerization of EGFR with other members of the EGFR family (Burden and Yarden, 1997; van der Geer et al., 1994). The transmembrane domain is a single 23-residue α-helical transmembrane peptide. This domain anchors the receptor to the plasma membrane. The juxtamembrane region of EGFR links the transmembrane domain to the kinase catalytic core. It has been shown to play an important role in regulatory functions. Several intrinsic sorting signals have been mapped to the EGFR juxtamembrane region (Hobert et al., 1997), including autonomous basolateral sorting determinants (Hobert et al., 1997; He et al., 2002), a lysosomal sorting signal (Kil et al., 1999; Kil and Carlin, 2000), and a nuclear localization signal (Lin et al., 2001). The intracellular domain also contains a 250-amino acid conserved protein tyrosine kinase core followed by a 229-residue carboxy-terminal tail with regulatory tyrosine residues (van der Geer et al., 1994). The tyrosine kinase domain provides the enzymatic activity and is responsible for downstream signaling. The carboxy terminal

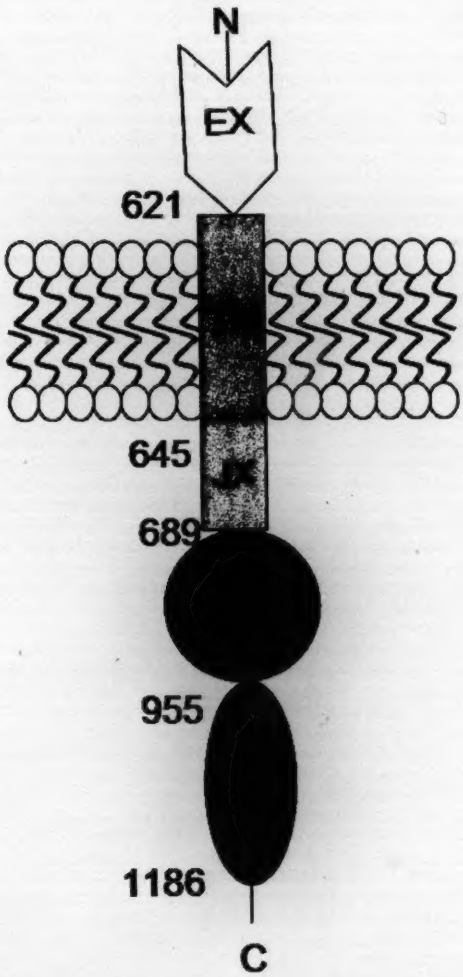


Figure 1.1 Schematic structure of EGFR with plasma membrane. Location of extracellular (EX), transmembrane (TM), juxtamembrane (JX), tyrosine kinase (TK), and carboxy-terminal (C-ter) domains in the EGFR are labeled with the residue numbers for domain boundaries.

regulatory domain becomes heavily phosphorylated and exerts both positive and negative effects upon receptor signaling (van der Geer et al., 1994).

Recent crystallographic studies of both the extracellular and intracellular domains of EGFR provide a better understanding of the molecular basis of EGFR signaling. In the extracellular region, two homologous leucine-rich (L) domains and two cysteine-rich domains are arranged in the order of L1-CR1-L2-CR2 (Ward et al., 1995). The CR domains contain several disulfide-bonded modules. It was initially proposed that L domains form the ligand-binding region and the CR domains mediate receptor dimerization and interactions with other membrane proteins (Lax et al., 1989). However, recent studies have suggested that the CR2 domain regulates ligand binding affinity by intramolecular interactions with the CR1 domain. The interactions between CR1 and CR2 domains lead to the auto-inhibition of the receptor in the absence of ligand, by constraining the relative orientation of L1 and L2 domains (Cho and Leahy, 2002). EGF binding is proposed to induce a significant rotation of the extracellular region of each receptor to expose the unique dimerization arms of CR1, hence promoting their interaction and subsequent trans-phosphorylation. In this sense, receptor dimerization is mediated entirely by receptor-receptor contacts and is not mediated by the bridging of EGF ligands (Cho and Leahy, 2002) (Figure 1.2).

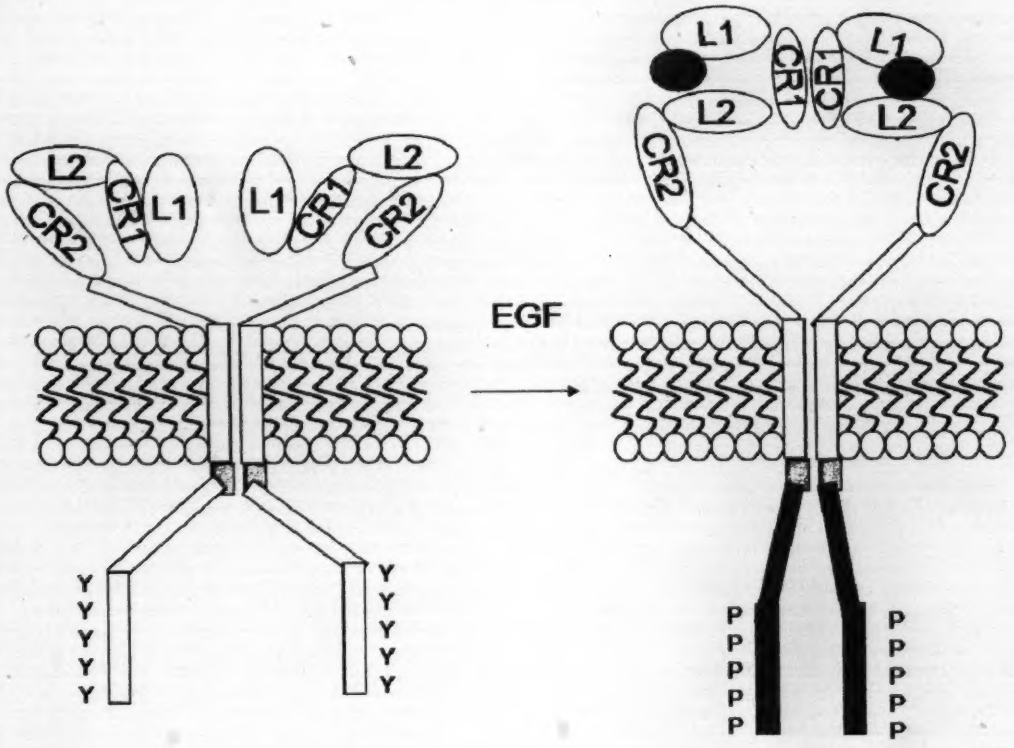


Figure 1.2 Suggested model of EGFR activation. The extracellular (EX) domain of EGFR is divided into the subdomains L1, CR1, L2, and CR2. In the inactive conformation of EGFR (left), each of the EX sub-domains are in a compact orientation and the tyrosine kinase (TK) domains are in the inactive form. Activation (right) occurs when EGF (red circles) bind to the EX domains, leading to a conformational change of the EX domain to form a stable dimer, which is followed by rotation of the transmembrane (TM) helices allowing dimerization. The TK domains also change orientations allowing them to phosphorylate tyrosine residues in the carboxy-terminal domain and other substrates (Cho and Leahy, 2002).

The crystal structure of the kinase domain is defined by the characteristic bilobate structure found in previously characterized protein kinase domains(Stamos et al., 2002; Hubbard and Till, 2000). The amino-terminal lobe (N-lobe) is formed by many β-strands and one α-helix, whereas the larger carboxyl-terminal lobe (C-lobe) is mostly α-helical. The two lobes are separated by an ATP-binding cleft. The important elements of the catalytic machinery surrounding the cleft include the glycine rich nucleotide-phosphate binding loop (Gly695 to Gly700) on the N-lobe. The C-lobe contains several essential components for catalysis, including the Mg-ATP binding motif (Asp831 to Gly833), the critical residue Asp813 in the catalytic loop (Arg812 to Asn818), and the activation loop (A-loop) (Asp831 to Val852) (Stamos et al., 2002; Hubbard and Till, 2000).

The structure of the carboxyl-terminal phosphorylation domain is the least characterized among the major domains of EGFR. The structure determined by Stamos *et al* shows the ordered carboxyl-terminal segments extending away from the C-lobe of the kinase (Stamos et al., 2002), whereas the kinase structure determined by Wood *et al* reveals the carboxyl-terminal segments interacting with the N- and C- lobes of the kinase domain (Wood et al., 2004). As such, the precise nature of the carboxyl-terminal conformation and its structural details need to be more thoroughly characterized.

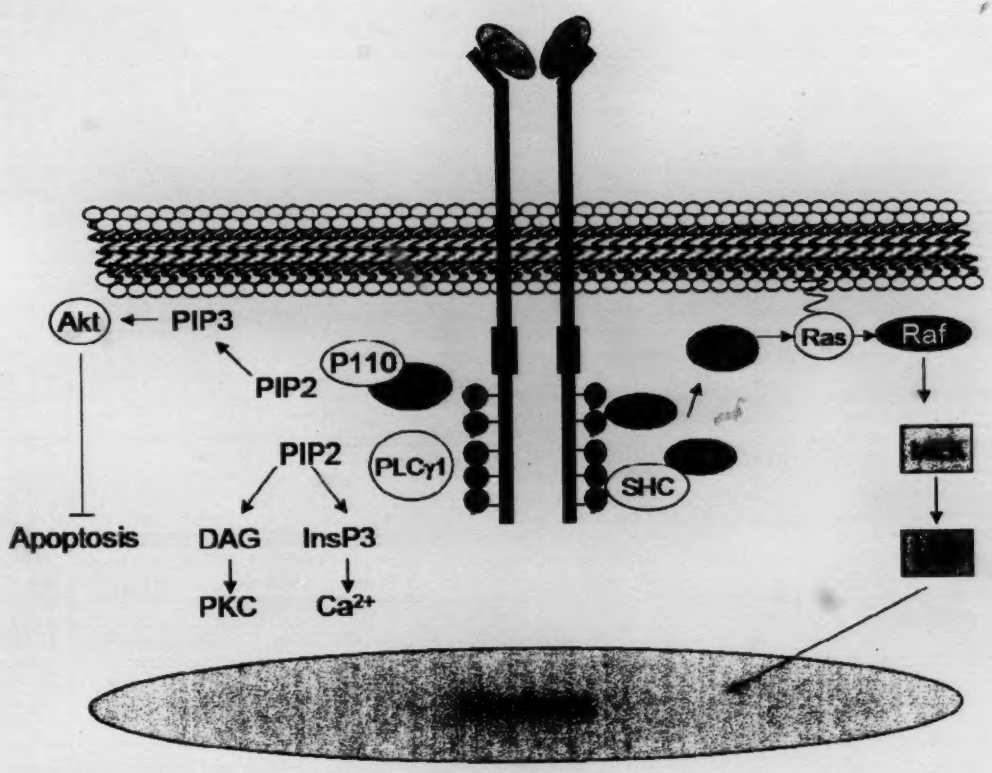


Figure 1.3 Activation of EGFR signaling cascades. Binding of EGF results in EGF receptor dimerization, autophosphorylation, and tyrosine phosphorylation of downstream proteins. The EGF receptor serves as a docking site for Grb2 and Sos, which in turn activate the Ras-Raf-MEK-ERK pathway, ultimately causing transcription of genes such as c-Fos and cell proliferation. PLC-γ1, which is activated by EGFR, hydrolyzes PIP2 to produce IP3 and DAG. IP3 causes calcium release from ER and DAG activates protein kinase C (PKC). EGFR also can activate Akt through PI3K kinase, which blocks apoptosis.

EGFR plays an important role in initiating signal transduction in cells to produce specific effects on cell growth and development (Schlessinger and Ullrich, 1992; Schlessinger, 2000). Binding of EGF at the plasma membrane induces dimerization of EGFR, which results in the activation of EGFR tyrosine kinase and trans-autophosphorylation (Carpenter, 1987; Schlessinger and Ullrich, 1992). Sites of tyrosine autophosphorylation in activated EGFR bind downstream signaling proteins to stimulate cell mitogenesis and block apoptosis through various signaling pathways including the PI3K-Akt, Ras-Erk and PLC-γ1 pathways (Pawson, 1995; Pawson, 1997; Schlessinger, 1986) (Figure 1.3).

1.3 The structure and function of PDGFR

Platelet-derived growth factor (PDGF) is a connective tissue cell mitogen that binds to the PDGF receptor (PDGFR) with high affinity (Hart et al., 1988; Heldin and Westermark, 1989). PDGF was first purified from human platelets, but is also expressed in a variety of other cell types, such as fibroblasts and smooth muscle cells. PDGF signaling is involved in a variety of processes, including embryogenesis, cell proliferation, cell differentiation, cell metabolism and cell motility (Reviewed in (Betsholtz, 2003)).

The PDGF family includes the four PDGF ligands (A, B, C, and D) as well as vascular endothelial growth factor (VEGF) (Li and Eriksson, 2003; Li et al., 2003). These ligands all have a PDGF/VEGF homology domain (80-90 amino acids) that contains eight conserved cysteines. The PDGFs form hetero- and homo- dimers (PDGF-AA,

PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD) via disulfide bonds (Oefner et al., 1992). This structure is conserved in a variety of growth factors with unrelated sequences including transforming growth factor β (TGFβ) and nerve growth factor (NGF) (Murray-Rust et al., 1993).

Thus far, two PDGF receptors have been identified (PDGFRα and PDGFRβ) (Kazlauskas and Cooper, 1989; Heldin et al., 1998). The PDGFRs, like their ligands, are also capable of forming homo- or heterodimers, depending on which of the ligand-dimers are bound (Figure 1.4). Like other RTKs, the PDGFR possesses an extracellular region (containing five immunoglobulin (IgG)-like domains), a single transmembrane segment, a juxtamembrane segment, a protein tyrosine kinase domain and a carboxyl-terminal tail (Heldin et al., 1998; Heldin and Westermark, 1999). IgG domains one to three are involved in the ligand binding, while IgG domain four is involved in dimer stabilization through receptor-receptor interactions. The transmembrane domain spans the plasma membrane and links the intracellular and extracellular domains.

Binding of PDGF to PDGFR induces the dimerization of PDGFR (Heldin and Westermark, 1989; Bishayee et al., 1989), which results in the activation of its intrinsic tyrosine kinase activity followed by trans-autophosphorylation (Ullrich and Schlessinger, 1990; Kelly et al., 1991). The phosphotyrosine recruits signaling proteins containing SH2 or PTB domains. Several of these signaling proteins have been identified, and include Src kinase family members (Kypta et al., 1990), PLC-γ1

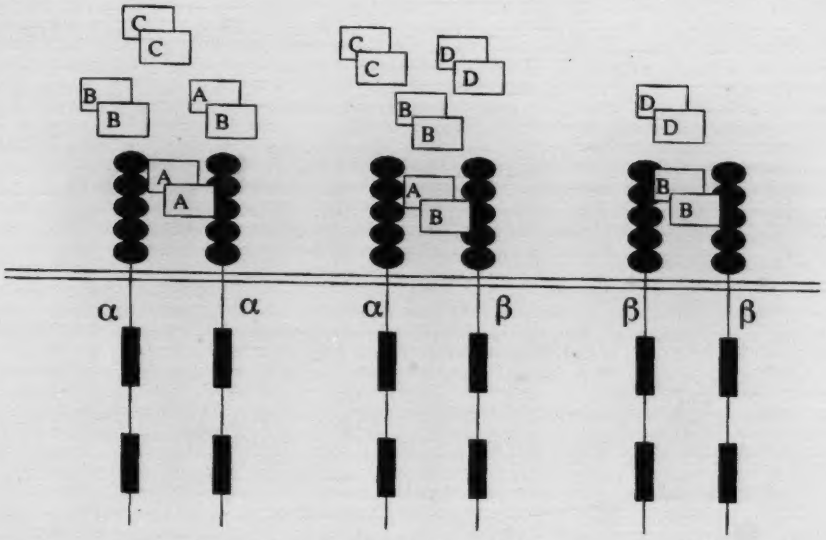


Figure 1.4. Schematic representing PDGFR and ligand binding partners. PDGFRα and PDGFRβ interact with the PDGF ligands to form receptor hetero- or homo-dimers. PDGFRα binds to PDGF-A, B, and C and PDGFRαα homodimer interacts with PDGF-AA, PDGF-BB, PDGF-AB and PDGF-CC. PDGFRβ binds to PDGF-B and D, and therefore PDGFRββ homodimer may only bind PDGF-BB and PDGF-DD. The PDGFRαβ heterodimer interacts with PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD.

(Meisenhelder et al., 1989; Wahl et al., 1990), the p85α subunit of phosphatidylinositol 3' kinase (PI3K) (Kazlauskas and Cooper, 1989), GAP (Fantl et al., 1992; Kashishian et al., 1992), the phosphotyrosine phosphatase SHP-2 (Lechleider et al., 1993; Kazlauskas, 1993), and adaptor proteins such as Grb2 (Rozakis-Adcock et al., 1992), SHC (Yokote et al., 1994), Grb7 (Stein et al., 1994) and Nck (Nishimura et al., 1993).The formation of receptor-signaling protein complexes initiates the activation of various signaling pathways including the Ras-Erk pathway, PI3K-Akt pathway, PLC-γ1 pathway and Src pathway. The activation of these pathways eventually leads to cell proliferation, survival and migration (Betsholtz, 2003; Heldin et al., 1998; Heldin and Westermark, 1990; Williams, 1989).

Like other RTKs, ligand binding also induces internalization of the ligand-receptor complex into endosomes. The PDGF-receptor complex then dissociates and either recycles back to the cell membrane or is degraded in the lysosomes (Takayama et al., 2005; Mori et al., 1994; Mori et al., 1991).

1.4 RTK-mediated signaling pathways

1.4.1 PI3K-Akt pathway

Recently, the PI3K-Akt pathway was shown to be involved in a variety of cell functions from cell motility, cell proliferation, cell differentiation, nutrient metabolism to anti-apoptosis. Disruption of the PI3K-Akt pathway causes cancer, and can lead to diabetes (Wymann et al., 1999; Toker, 2000; Toker and Newton, 2000). Three PI3K classes have been defined on the basis of their primary structure, regulation and *in vitro*

lipid substrate specificity. Class I PI3Ks produce phosphatidylinositol 3-phosphate (PtdIns(3)P), phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) and phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3), and are activated by RTKs and G-protein-coupled receptors. Class II PI3Ks generate PtdIns(3)P and PtdIns(3,4)P_2 and possess a C2 domain at their carboxyl-terminus which is implicated in lipid binding. Class III PI3Ks produce PtdIns(3)P only. The mechanism of Class II and Class III PI3Ks activation is still obscure.

The class I PI3K is composed of an 85 kD regulatory subunit and a 110 kD catalytic subunit, which transfers the γ-phosphate group of ATP to the D-3 position of the inositol headgroup. The p85 regulatory subunit contains a N-terminal SH3 domain, two proline-rich regions, a Bcr homology domain, and two carboxy-terminal SH2 domains spaced by an inter-SH2 region. The inter-SH2 region mediates tight binding of p85 to the catalytic subunit. PI3Ks are activated following the binding of their SH2 domains to pTyr residues of the activated RTKs or their substrates (insulin receptor substrates). The lipid products of PI3K can activate several downstream targets, such as PI-dependent kinases (PDK1), PDK2 and Akt (Protein kinase B, PKB). In addition, PI3K was proposed to function as a scaffold to recruit different downstream proteins, such as Cdc42, Rac1, Arc, Abl, Lyn, Fyn, SHC and others (Wymann et al., 1999; Toker, 2000; Toker and Newton, 2000) .

Akt is a very important effector of PI3K. Akt is composed of an amino terminal PH domain, a central kinase domain and a carboxyl terminal regulatory domain (Figure 1.5).

The PH domain of Akt binds PtdIns(3,4,5)P_3, which is the lipid product of PI3K upon growth factor stimulation. This binding induces the translocation of Akt from cytosol to the plasma membrane, what it is then activated by the PDK1/2. All three Akt isoforms have carboxyl terminal extensions of around 40 amino acids. This region possesses the FXXF/Y-S/T-Y/F hydrophobic motif. In mammalian Akt isoforms, this motif is identical (FPQFSY). Deletion of this motif completely abolishes the enzymatic activity of Akt (Andjelkovic et al., 1997). GFs activate Akt by activating PI3K (Hanada et al., 2004). GF-induced phosphorylation of T308 and S473 is essential for Akt activity. Point mutantations of these amino acids to Ala show little activity and the phosphorylation mimicking mutants (T308D/S473D) show constitutive kinase activity (James et al., 1996). It was recently reported that Akt could be activated by the phosphorylation of its tyrosine residues, including Tyr315 and 326. These sites were phosphorylated by c-Src, and mediated by the interaction of Src SH3 domain with the PXXP motif in the carboxy-terminal of Akt in an EGF-dependent manner (Chen and Wang, 2001). The minimal substrate consensus sequence for Akt is RXRXXS/T.

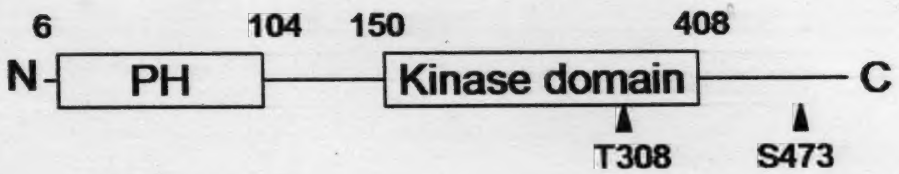


Figure 1.5 Schematic structure of Akt. Akt is composed of an amino terminal PH domain, a central kinase domain and a carboxyl terminal regulatory domain. Phosphorylation at Thr-308 in the catalytic loop and Ser-473 in the carboxyl terminal regulatory domain are required for full activation of Akt.

Akt activation triggers a diverse array of cellular responses. The best-defined function of Akt in GF-induced cell signaling is to regulate apoptosis. Akt promotes cell survival by phosphorylating the proapoptotic factors BAD S136 (Datta et al., 1997) and procaspase-9, as well as the Forkhead family of transcription factors that induce the expression of proapoptotic factors such as Fas ligand. Recently, Akt activation has been implicated in increased cell resistance to apoptosis induced by TNF-Related Apoptosis-Inducing Ligand (TRAIL), a member of the TNF superfamily that has been shown to have selective anti-tumor activity (Zauli et al., 2005; Park and Seol, 2002; Wang, 2002).

Akt can also modulate glycogen metabolism through glycogen synthase kinase-3 (GSK-3), mammalian target of rapamycin (mTOR) and insulin receptor substrate-1 (IRS-1). Recently, Akt was also suggested to regulate cell motility and cancer cell invasion (Yoeli-Lerner and Toker, 2006; Enomoto et al., 2005; Higuchi et al., 2001; Kim, 2001; Cheng et al., 2000).

1.4.2 Ras-Raf-MEK-ERK pathway

The Ras-Raf-MEK-ERK pathway mediates numerous cellular functions in different tissues and cell types such as cell cycle progression (Kerkhoff and Rapp, 1998b; Kerkhoff and Rapp, 1998a; Kerkhoff et al., 1998; Lavoie et al., 1996a; Lavoie et al., 1996b; Stacey and Kazlauskas, 2002), differentiation (Nishida et al., 1996; Rommel et al., 1999; Sternberg et al., 1993; Wood et al., 1993), transformation (Fukui et al., 1985; Heidecker et al., 1990; Stanton, Jr. and Cooper, 1987), anti-apoptosis (Lau et al., 1998;

Mikula et al., 2001) and proliferation (Chadee and Kyriakis, 2004b; Chadee and Kyriakis, 2004a; Zhang et al., 2004)

Grb2 is an adaptor protein that contains one SH2 and two SH3 domains. Grb2 SH2 domain binds to RTKs either directly or indirectly through another adaptor protein, SHC. Grb2 SH3 domains interact with son-of sevenless (SOS), a guanine nucleotide exchange factor (GEF) of Ras. Recruitment of Grb2/Sos to the plasma membrane results in the activation of Ras and the subsequent activation of the Raf-MEK-ERK signaling pathway (Downward, 1994; Wang and Moran, 1996),

Ras is a small GTPase that plays a central role in this signaling pathway. Following its activation by SOS, activated Ras recruits the serine-threonine kinase Raf to the inner leaflet of the plasma membrane and induces activation of its kinase domain (Cook and McCormick, 1993; Wotton et al., 1993; Williams et al., 1993; Dickson et al., 1992a; Troppmair et al., 1992; Wood et al., 1992; Van Aelst et al., 1993; Zhang et al., 1993; Dickson et al., 1992b; Warne et al., 1993; Vojtek et al., 1993).

Raf is a serine/threonine specific kinase, comprising a family of three isoforms, A-Raf, B-Raf and Raf-1. All Raf kinases are composed of three conserved regions, CR1, CR2 and CR3 (Morrison and Cutler, 1997). The initial process of Raf activation involves the interaction of active GTP-bound Ras with the Ras binding domain (RBD) and cysteine rich domain (CRD) of CR1, and subsequent recruitment of Raf to the membrane for further activation (Morrison and Cutler, 1997; Vojtek et al., 1993). The role of the CR2 region is less well defined, however. Phosphorylation of CR2 and various

protein-protein interactions via CR2 appear to affect the localization and activation of Raf (Morrison et al., 1993; Guan et al., 2000; Yao et al., 1995; Zhang and Guan, 2000; Zimmermann and Moelling, 1999). CR3 is the catalytic kinase domain of Raf and is subject to regulation by phosphorylation.

Activated Raf phosphorylates and activates mitogen-activated protein kinase (MAPK) kinase (MEK) (Dent et al., 1992; Howe et al., 1992; Kyriakis et al., 1992). MEK belongs to the rare breed of dual specificity kinases which can phosphorylate both threonine and tyrosine residues. It phosphorylates p44 and p42 extracellular signal-regulated kinase (ERK, also as mitogen-activated protein kinase (MAPK)) via phosphorylation of a Thr-Glu-Tyr motif in the activation loop (Moodie et al., 1993; Lange-Carter et al., 1993). ERK is a serine/theronine kinase with an impressive portfolio of more than 50 substrates, and its primary function is to modulate the activity of transcriptional factors c-Fos and c-Jun (Mulcahy et al., 1985; Stacey et al., 1987; Marshall, 1994) (Fig 1.3).

1.4.3 PLC-γ1 pathways

PLC-γ1 pathway has been implicated in many GF-induced cell signaling outcomes including cell proliferation, differentiation, receptor endocytosis, cell motility, membrane ruffle formation and branching tubulogenesis (Wahl and Carpenter, 1991; Kamat and Carpenter, 1997; Wells et al., 1998;Gual et al., 2000; Meyer et al., 2003; Bivona et al., 2003; Chou et al., 2003; Choi et al., 2004). Overexpression and hyperactivation of PLC-γ1 have been implicated in many types of tumor cells, including colorectal, prostate,

breast, cervical, and ovarian (Turner 1997; Kassis 1999; Khoshyomn 1999; Wells 2000). Overexpression of PLC-γ1 is also linked to tumor aggression (Thomas et al., 2003;Wells and Grandis, 2003). PLC-γ1 forms a complex *in vivo* with RTK through its SH2 domain interaction in response to GF stimulation (Wahl et al., 1988; Margolis et al., 1989; Meisenhelder et al., 1989; Anderson et al., 1990; Margolis et al., 1990). Complex formation leads to phosphorylation of PLC-γ1 on tyrosine residues and an increase in its enzymatic activity (Kim et al., 1991; Rotin et al., 1992; Ronnstrand et al., 1992). Active PLC-γ1 hydrolyzes PtdIns(4,5)P_2 to form the second messengers inositol 1, 4, 5-triphosphate (InsP3) and diacylglycerol (DAG), which mobilizes intracellular calcium and activates protein kinase C (PKC), respectively (Wahl and Carpenter, 1991).

The sequence of PLC-γ1 contains a string of domains including a N-terminal PH domain, a split PH domain, EF-hand motifs, a catalytic α/β barrel forming the characteristic X- and Y- box regions, two SH2 domains, one SH3 domain, and a single C2 domain (Figure 1.6). The structure of PLC-γ1 has not been determined, but the crystallographic data from its structurally similar isoform PLC-δ1 suggests that this domain is comprised of alternating α-helices and β-strands, resembling an incomplete triose phosphate isomerase (TIM). The catalytic residues are located at one end of the β-barrel. The PLC-γ1 has four EF-hand motifs, each consisting of a helix-loop-helix structure. Unlike its analogue in yeast and *D. discoideum*, the EF-hand lack residues critical to calcium binding and fails to bind calcium. The regulatory function of the EF-hand has yet to be determined. The C2 domain is 120 residues in length and consists

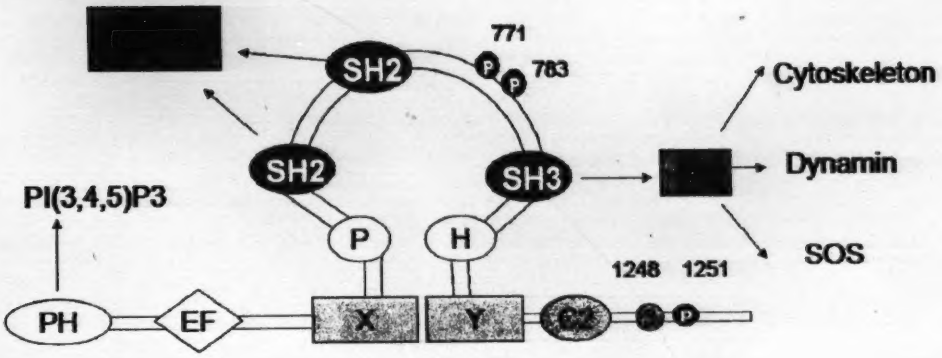


Figure 1.6 The modular domains of PLC-γ1. PLC-γ1 contains a N-terminal PH domain, a split PH domain, EF-hand motifs, a catalytic X- and Y- box region, two SH2 domains, one SH3 domain, and a single C2 domain. It also contains three major tyrosine phosphorylation sites, pY771, pY783, pY1251 and one serine phosphorylation site pS1248.

of eight antiparallel β-strands arranged as a sandwich. The C2 domain is involved in calcium binding. The PH, SH2 and SH3 domains are involved in the localization of PLC-γ1 and will be discussed later (Figure 1.6).

GF stimulates the translocation of PLC-γ1, playing an important role in modulating the function of PLC-γ1. However the translocation pattern of PLC-γ1 following GF stimulation is still not clear. Initially, it was suggested that PLC-γ1 translocates from the cytosol to the plasma membrane to form a complex with growth factor receptors (Todderud et al., 1990). Recently it was shown that green fluorescent protein (GFP) tagged PLC-γ1 translocated to the endosomes following its initial translocation from cytosol to the plasma membrane in response to EGF (Matsuda et al., 2001). However, another recent report showed that PLC-γ1 translocated to the nucleus following NGF stimulation (Ye et al., 2002). Thus, it is not clear whether GF stimulates the translocation of PLC-γ1 to endosomes, nucleus or both.

PLC-γ1 contains two SH2 domains, one SH3 domain, one split PH domain and one complete PH domain (Figure 1.6). Each of these domains can fold into a compact and functional module independent of surrounding sequences (Pawson, 1995). All of these domains have been implicated in controlling localization and GF-induced translocation of PLC-γ1. Initially it was shown that the translocation of PLC-γ1 from cytosol to the plasma membrane is regulated by the interactions between the SH2 domains of PLC-γ1 and the phosphotyrosine residues of GF receptors (Matsuda et al., 2001). Recently, it was reported that overexpression of the PLC-γ1 PH domain prevents PLC-γ1 membrane

targeting (Falasca et al., 1998). The PH domain of PLC-γ1 binds to PtdIns(3,4,5)P_3, and is targeted to the membrane in response to growth factor stimulation. Consistent with these observations, activation of PI3-kinase causes PLC-γ1 PH domain-mediated membrane targeting and PLC-γ1 activation (Falasca et al., 1998). However, a recent publication showed that the translocation of PLC-γ1 to the plasma membrane required functional SH2 domains and was not affected by mutations in the PH domain or inhibition of PI3K (Matsuda et al., 2001). These data are directly at odds with the finding of Falasca *et al.*.

The issue regarding the regulation of GF-induced translocation of PLC-γ1 is further complicated by the following findings. It has been reported that the C-SH2 domain of PLC-γ1 binds to F-actin (Pei et al., 1996), tubulin (Itoh et al., 1996) and PtdIns(3,4,5)P_3 (Bae et al., 1998) *in vitro*, which suggests that the C-SH2 domain may be involved in the localization of PLC-γ1 to the cell cytoskeleton. PLC-γ1 SH3 domain has been shown to target PLC-γ1 to the cytoskeleton and may function to regulate actin polymerization (Bar-Sagi et al., 1993). Thus, another intriguing question is which domain-SH2, SH3 or PH domain, is responsible for regulating the translocation of PLC-γ1 in response to GF.

The role of PLC-γ1 in GF induced mitogenesis has been supported by various studies. Most of the studies indicate that the mitogenic property of PLC-γ1 is dependent on its interaction with RTKs and on the activation of its enzymatic activity (Watt et al., 2002). It has been reported that injection of antibodies against PLC-γ1 blocks serum- and Ras-stimulated DNA synthesis, which indicates that PLC-γ1 is required for

serum-induced mitogenesis and that PLC-γ1 is downstream of Ras (Smith et al., 1990). Another report has showed that inhibition of Ras activity with N17Ras has no effect on GF-induced activation of PLC-γ1 (Troppmair et al., 1992). It has been shown that PLC-γ1 is required for PDGF-induced DNA synthesis and c-Fos expression (Roche et al., 1996) and homozygous disruption of the PLC-γ1 gene *Plcγ1* results in embryonic lethality at approximately embryonic day (E) 9.0 (Ji et al., 1997). Moreover, PLC-γ1 enzymatic activity is required for GF-induced mitogenesis and this function may be to produce DAG and subsequently to activate PKC.

PLC-γ1 is likely involved in much broader cell signaling than previously revealed. Interestingly, most of the recently identified interactions between PLC-γ1 and its binding proteins are mediated by its SH3 domain. Four examples exist to support the importance of this interaction. Firstly, EGF stimulates the interaction between PLC-γ1 and PLD$_2$ which is mediated by the PLC-γ1 SH3 domain to potentiate EGF-induced IP$_3$ formation and Ca^{2+} increase (Haugh and Meyer, 2002). Secondly, PLC-γ1 SH3 domain is essential for the activation of calcium entry into cells after stimulation of cell-surface receptors (Patterson et al., 2002). Thirdly, PLC-γ1 SH3 domain acts as a guanine nucleotide exchange factor (GEF) for PIKE to regulate NGF-induced cell mitogenesis (Ye et al., 2002). Lastly, PLC-γ1 SH3 domain acts as a GEF for dynamin1 to regulate EGFR endocytosis. Here, the interaction between PLCγ-1 SH3 domain and dynamin1 is EGF-dependent (Choi et al., 2004).

It has been reported that PLC-γ1 is required for cell motility induced by many GFs,

including EGF (Chen et al., 1994), PDGF (Kundra et al., 1994), and HGF (Derman et al., 1996). While the mechanisms by which PLC-γ1 regulates cell motility is not clear, it was suggested that activation of PLC-γ1 by EGF resulted in the reorganization of the cytoskeleton (Wells et al., 2002). The role of PLC-γ1 in the regulation of cell motility has been shown in a variety of cell types, especially carcinoma cells (Wells et al., 2002).

It is well-established that activated EGFR directly stimulates PLC-γ1 by phosphorylating its tyrosine residues and indirectly stimulates Akt by activating PI3K (Carpenter and Ji, 1999). Both PLC-γ1 and PI3K-Akt pathways play a significant role in EGFR-mediated cell signaling. While both PLC-γ1 and PI3K-Akt play distinct roles in cell signaling, the two pathways have been shown to interact with each other. For example, PLC-γ1 and PI3K are both involved in lipid metabolism and use the same substrate-PtdIns(4,5)P_2 (Carpenter and Ji, 1999). Moreover, both PLC-γ1 and PI3K-Akt pathways are involved in cell mitogenesis and motility (Chen et al., 1994). It is known that PLC-γ1 may regulate Ras activity through the activation of rasGRP and activated Ras then stimulates the activation of PI3K (Ehrhardt et al., 2004). However, it is not known whether PLC-γ1 directly binds to Akt.

1.5 Regulation of RTK-mediated endocytosis

Cells must posses the ability to terminate EGFR signaling. Several mechanisms were identified for this process, such as tyrosine dephosphorylation by tyrosine phosphotases, serine and theronine phosphorylation, and ligand induced endocytosis

(Schlessinger, 2000; Pawson et al., 2002). Concomitant with its activation of various signaling pathways, binding of EGF also rapidly triggers the clustering and internalization of the ligand-receptor complexes via coated pits and coated vesicles (Schlessinger, 1986; Carpenter, 1987). Receptor-mediated endocytosis occurs in two stages and each of them can be divided into multiple steps (Goldstein et al., 1985): 1) internalization, which covers the steps from targeting of receptors to coated pits to the formation of coated vesicles and 2) intracellular receptor trafficking, covering the steps following receptor internalization (Figure 1.7). Although the exact mechanism has not been determined, it is known that several proteins or protein complexes, including adaptin AP-2, clathrin and dynamin participate in the internalization of all receptor tyrosine kinases. Adaptin AP-2 is a cytoplasmic protein complex that interacts with the cytoplasmic tails of various receptors (Robinson, 1994). These interactions are thought to account for the ability of the cell to selectively direct receptors to clathrin-coated vesicles (Zaremba and Keen, 1983). The clathrin molecule is composed of three heavy chains and three light chains, joined together to form a three-legged assembly called a triskelion. The cell then utilizes these triskelion modules to form polygonal scaffolds. These scaffolds, when attached to a membrane, cause the membrane to deform into a budding vesicle. Clathrin binds to the membrane by interacting with membrane-bound adaptin AP-2 (Robinson, 1994). Dynamin is composed of three GTP binding elements at its N-terminus

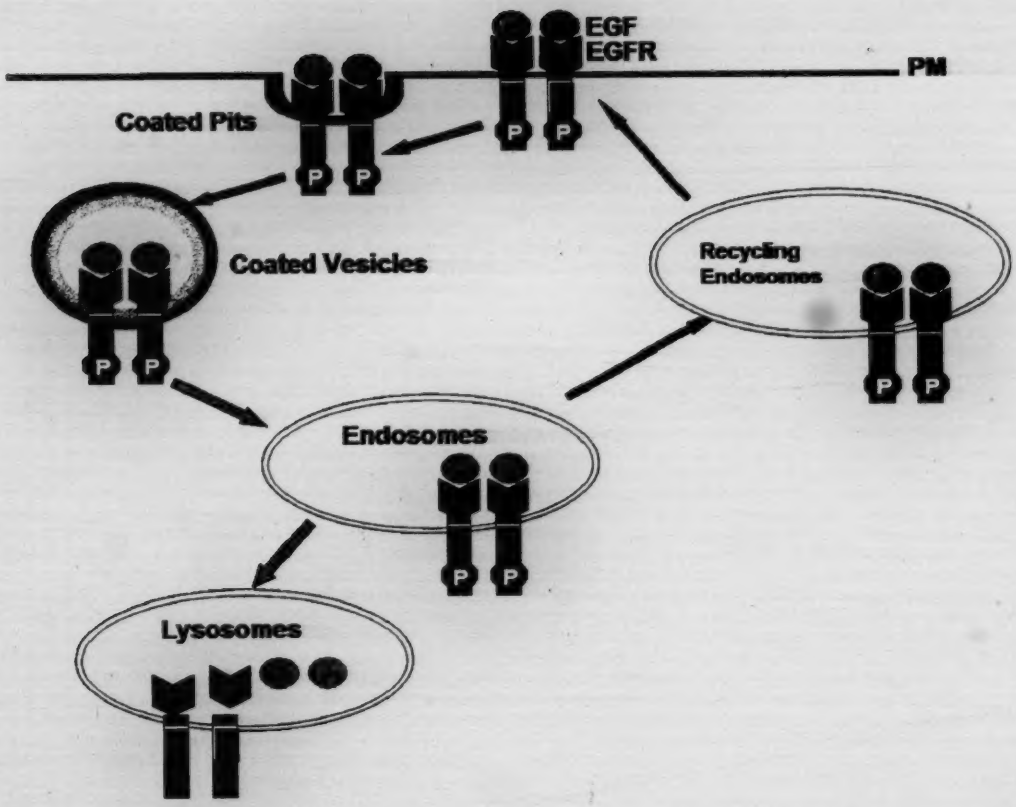


Figure 1.7 Schematic demonstration of EGFR endocytosis. Receptor mediated endocytosis is a multi-step event, utilizing coated pits, coated vesicles, endosomes and lysosomes. Most EGF-EGFR complexes are proteolytically degraded in lysosomes, while some recycle back to the cell surface.

and two overlapping consensus sequences for SH3 domain binding at the carboxyl-terminus, separated by a PH domain. Expression of dominant-negative dynamin blocks vesicle formation. (Herskovits et al., 1993; Damke et al., 1994).

Intracellular trafficking of receptors involves a series of membrane budding and fusion events (Rothman, 1994). These are regulated by specific cytosolic and membrane associated protein factors, including two groups of Ras-like small guanosine triphosphatases (GTPases) called Rabs (Valencia et al., 1991) and adenosine diphosphate (ADP)-ribosylation factors (ARFs) (Donaldson et al., 2005; Clark et al., 1993; Tsuchiya et al., 1991). The current view is that Rabs are involved in the specification of the correctness of membrane-membrane interactions at the level of docking and fusion, or both (Zerial and Stenmark, 1993). For example, both Rab4 and Rab5 localize to early endosomes. Rab4 controls the function and formation of endosomes involved in recycling (van der Sluijs et al., 1992a; van der Sluijs et al., 1992b; Bottger et al., 1996b). On the other hand, Rab5 controls membrane trafficking in the early endocytic pathway (Bucci et al., 1992; Stenmark et al., 1994; Rybin et al., 1996). Another interesting Rab is Rab7, which functions downstream of Rab5 and regulates membrane transport leading from early to late endosomes or to lysosomes (Feng et al., 1995; Bottger et al., 1996a). The fate of internalized EGF and EGFRs is primarily proteolytic degradation in lysosomes, while 15-25% of the occupied EGFR escapes back to the cell surface (Carpenter, 1987).

Binding of EGF results in the clustering and internalization of EGFR. EGF and EGFR can be detected in early endosomes after 1-5 min incubation with EGF at 37°C, in

late endosomes after 20 min at 37°C, and in lysosomes after 40-60 min 37°C.

1.6 Regulation of RTK-mediated signal transduction by endocytosis

Endocytosis is always thought as one of the important degradation pathways of EGFR. Whether the internalization leads exclusively to receptor inactivation or is important in triggering or amplifying cellular responses to growth factors, however, remain important questions (Di Fiore and Gill, 1999; Di Fiore and De Camilli, 2001; Ceresa and Schmid, 2000). It is well established that endocytosis of EGFR from the cell surface to lysosomes results in the degradation of the receptors, which can attenuate receptor signaling and may even be conceived of as a tumor suppression pathway (Di Fiore and Gill, 1999). On the other hand, accumulated evidence suggests that internalized EGF-EGFR complex maintains its ability to generate cell signaling from endosomes. Internalized EGFRs are autophosphorylated and catalytically active (Kay et al., 1986; Lai et al., 1989). Various signaling molecules that regulate Ras activities, including Grb2, SHC and GAP, are co-internalized with EGFR into endosomes and remain associated with EGFR in endosomes (Di Guglielmo et al., 1994; Wang et al., 1996). Recently, more results confirmed the interaction between EGFR and various signaling proteins in endosomes (Haugh et al., 1999; Kuruvilla et al., 2000; Burke et al., 2001; Slepnev and De Camilli, 2000; Miller and Lefkowitz, 2001).

The strongest evidence supporting the signaling endosome hypothesis comes from endocytosis blocking experiments. Inhibition of EGFR endocytosis by a mutant dynamin results in enhanced cell proliferation but decreased MAPK activation (Vieira et al., 1996).

In a recent study of transactivation of EGFR by G-protein coupled receptors, it was found that inhibition of EGFR endocytosis by mutant dynamin or β-arrestin abolished MAPK activation (Ahn et al., 1999). Inhibition of EGFR endocytosis by phospholipase D also blocks EGF stimulated-MAPK activation (Shen et al., 2001). However, this endocytosis-inhibition approach has serious limitations in studying endosomal signaling. First, while inhibition of EGFR endocytosis eliminates endosomal signaling, it prolongs the signaling from the plasma membrane. Thus, it is difficult to determine whether the observed effects are due to the lack of endosomal signaling or due to prolonged plasma membrane signaling. Second, blocking of EGFR endocytosis by mutant dynamin, clathrin or β-arrestin globally affects endocytosis mediated by dynamin, clathrin or β-arrestin. Thus, it is difficult to determine whether the observed effects are due to the inhibition of EGFR endosomal signaling or due to the broad inhibition of endocytosis. Third, this approach is not suitable for studying the dynamics of endosomal signaling.

In spite of intensified efforts in understanding cell signaling from endosomes (Haugh et al., 1999; Kuruvilla et al., 2000; Burke et al., 2001; Slepnev and De Camilli, 2000; Miller and Lefkowitz, 2001), many questions remain unanswered due to the limitations of current approaches. There is no direct evidence to demonstrate whether any meaningful signals are solely generated from endosomes by internalized EGFRs. It is not clear what kinds of signals, if any, are generated from endosomes and what the consequences of these signals are on cell mitogenesis and anti-apoptosis. The lack of breakthrough in this aspect is due to the lack of a system by which EGFR can be

selectively activated in endosomes.

1.7 Rationales, hypotheses and objectives

RTK-mediated signal transduction is a central component of cell signaling networks and plays a crucial role in normal physiological processes, such as embryogenesis, cell proliferation, cell differentiation, cell metabolism, cell death (apoptosis) and cell motility. Unregulated activation of RTK-mediated cell signaling through mechanisms such as point mutations, over-expression or aberrant cellular localization of the signaling molecules can lead to various forms of cancer and other diseases. Due to the essential roles of RTK and its signaling, various mechanisms have been established in the cell to assure the precise and tight control of RTK signaling. These mechanisms include the modulation of its kinase activity by specific phosphorylation and dephosphorylation of Tyr/Ser/Thr residues, spatial regulation of various signaling pathways by translocation to various intracellular compartments such as endosomes and the plasma membrane, and regulation of specific cell function by selective interaction with specific downstream signaling molecules.

My research covers two aspects of these regulatory mechanisms. I first studied the poorly understood spatial regulation of RTK signaling. Especially, I focused my study on the physiological relevance of endosomal signaling of RTKs.

For more than a decade, investigators have debated the existence of endosomal signaling. Initially, endocytosis of ligand-activated receptors was generally considered a mechanism to attenuate signaling. Recently, more evidence has suggested that

internalized receptors may maintain their ability to generate cell signaling from endosomes. Strong evidence supporting endosomal signaling comes from endocytosis inhibition experiments. However, in spite of intensified efforts to understand cell signaling from endosomes, it has been argued that receptor-mediated signal transduction is primarily restricted to the cell surface. The lack of breakthrough is due to the limitation of current approaches. None of the approaches used to date offer a means to generate pure endosomal signaling and so far, no research has been able to demonstrate the physiological relevance of endosomal signaling. **My hypothesis is that endosomal signaling of EGFR is sufficient to stimulate cell mitogenesis and anti-apoptosis, and thus, the endosome is a physiologically important center for cell signaling.** In this thesis, I tested this hypothesis by establishing a cell system that specifically generates endosomal RTK signaling in the absence of cell surface RTK signaling.

The second research area in my thesis is the regulation of RTK-mediated cell signaling by PLC-γ1. RTK-mediated cell signaling is regulated and mediated by various signaling proteins. The role of PLC-γ1 is still poorly understood. Recent results from ours and several other laboratories suggest that PLC-γ1 plays a much broader and more complicated role in cell signaling. This is in part due to the multi-domain nature of PLC-γ1. Indeed, all PLC-γ1 domains, including SH2, SH3, PH and phospholipase domains, have been implicated in GF-induced cell signaling. However, the roles of PLC-γ1 in various RTK-mediated cell signaling events are still very controversial and the molecular mechanism by which PLC-γ1 regulates RTK-mediated cell signaling is far

from clear. **My hypothesis is that PLC-γ1 regulates broad cell signaling mediated by RTKs through its multiple domains, which allows a broad interaction with various proteins and lipids.** In this thesis, I studied the regulation of PLC-γ1 translocation in response to RTK activation and identified a novel interaction between PLC-γ1 and Akt.

Chapter 2

Materials and Methods

2.1 Materials

All chemicals, enzymes and kits were used according to the manufacturers' specifications and in accordance with protocols set out by the Environmental Health and Safety of the University of Alberta and Workplace Hazardous Materials Information System (WHMIS).

2.1.1 Chemicals and Reagents

acetic acid, glacial	BDH
acrylamide/bis	Biorad
AEBSF	Sigma
AG1296	Calbiochem
AG1478	Calbiochem
agar	Gibco
agarose	Gibco
ammonium persulfate	BDH
aprotinin	Sigma
bacto-tryptone	Difco
β-mercaptoethanol	Sigma
bromodeoxy uridine (BrdU)	Amersham
bromophenol blue	Biorad
buria-Bertani media, broth base	Gibco
calcium chloride	Sigma
coomasie brilliant blue, G250	Biorad
dimethyl sulfoxide	Fisher
disulfosuccinimidyl suberate (DSS)	Calbiochem
dulbecco's modified eagle medium (DMEM)	Gibco
epidermal growth factor (EGF)	Upstate
ethanol, 95%	Fisher
ethidium bromide	OmniPur
ethylene glycol-bis (b-amino ethyl ether tetra acetic acid) (EGTA)	Sigma
fetal bovine serum	Sigma
glucose	EM science
glycerol	BDH
glycine	Biorad

hydrochloric acid	Fisher
isopropanol	Fisher
kanamycin	Sigma
magnesium chloride	BDH
monensin	Calbiochem
N,N-bis[2hydroxyethyl]-2-aminoethanesulfonic acid (BES)	Sigma
nonidet P40	BDH
non-essential amino acids	Invitrogen
PDGF-B	Upstate
pepstatin a	Sigma
phosphate buffered saline, 10x	OmniPur
potassium chloride	BDH
sodium chloride	BDH
sodium azide	Sigma
sodium dodecyl sulfate (SDS)	Biorad
sodium fluoride	Sigma
sodium orthovanadate	Sigma
sucrose	Biobasic
tetramethylethylenediamine (TEMED)	Gibco
tris (hydroxymethyl) aminomethane	Biorad
Triton X-100	BDH
Tween 20	Fisher
yeast extract, select	Gibco

2.1.2 Other Materials

Medical X-ray Film	Fuji
Transblot Nitrocellulose	Biorad
Whatman Chromatography Paper	Fisher
glutathione insolubilized on cross-linked 4% agarose	Sigma
goat anti-mouse IgG conjugated with agarose	Sigma
Protein A sepharose 6MB	Pharmacia

2.1.3 Enzymes

DNA ligase, T4	Gibco
Hot Start Taq, DNA polymerase	Qiagen
dNTPs	Invitrogen
Restriction endonucleases	Gibco
RNAase	Sigma

2.1.4 Experimental Kits

QIAprep Spin Mniniprep Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
HiSpeed Plasmid Midi Kit	Qiagen
Supersignal ECL Western Blotting Detection Reagents	Pierce
Topo-XL PCR cloning kit	Invitrogen

2.1.5 Plasmids

pCR-XL-TOPO	Invitrogen
pDsRed-C1	Clontech
pEGFP-N3	Clontech
pEYFP-C1	Clontech

2.1.6 Antibodies

2.1.6.1 Primary Antibodies

Mouse anti-phospho-serine antibody	Sigma
Mouse anti-BrdU	Amersham
Mouse anti-EEA1	BD Pharmingen
Mouse anti-EGFR antibody (Ab1)	Sigma
Mouse anti-GST antibody	Santa Cruz
Mouse anti-lamin	American Research Products
Mouse anti-phopho-ERK1/2	Santa Cruz
Mouse anti-phospho-PLC-γ1 (Tyr 783)	Medicore
Mouse anti-phospho-tyrosine (py99)	Santa Cruz
Mouse anti-PLC-γ1	Upstate
Mouse anti-tubulin	Santa Cruz
Mouse anti-Ras	Upstate
Rabbit anti-ERK	Santa Cruz
Rabbit anti-C-Cbl	Santa Cruz
Rabbit anti-EGFR(1005)	Santa Cruz
Rabbit anti-GFP	Clonetech
Rabbit anti-Grb2	Santa Cruz
Rabbit anti-PDGFR	Santa Cruz
Rabbit anti-phosphoserine (1248) of PLC-γ1	Upstate
Rabbit anti-phospho-EGFR (py1068)	Santa Cruz
Rabbit anti-phospho-EGFR (py1086)	Santa Cruz

Rabbit anti-phospho-EGFR (py1148)	Santa Cruz
Rabbit anti-phospho-EGFR (py1173)	Santa Cruz
Rabbit anti-phospho-EGFR (py992)	Santa Cruz
Rabbit anti-phospho-PLC-γ1 (Tyr 783)	Santa Cruz
Rabbit anti-phospho-PDGFR	Santa Cruz
Rabbit anti-PLC-γ1	Santa Cruz
Rabbit anti-Raf	Santa Cruz
Rabbit anti-SHC antibody	BD Transduction laboratory
Rabbit polyclonal anti-phospho-Akt (ser 473)	Cell signaling
Rabbit polyclonal anti-p85	Upstate
Rabbit polyclonal anti-phospho-c-Jun (ser 653)	Cell signaling

2.1.6.2 Secondary Antibodies

Rhodamin-conjugated anti-mouse	Jackson Immunoresearch
Rhodamin-conjugated anti-rabbit	Jackson Immunoresearch
Rhodamin-conjugated anti-mouse	Jackson Immunoresearch
Rhodamin-conjugated anti-mouse	Jackson Immunoresearch
Horseradish peroxidase(HRP)-conjugated anti-rabbit	Bio-Rad
Horseradish peroxidase(HRP)-conjugated anti-mouse	Bio-Rad
Horseradish peroxidase(HRP)-conjugated anti-sheep	Bio-Rad
Horseradish peroxidase(HRP)-conjugated anti-goat	Bio-Rad

2.1.7 Molecular Size Markers

100bp DNA ladder	Gibco
1kb DNA ladder	Gibco
Prestained markers for SDS-PAGE	Sigma

2.1.8 Buffers and Other Solutions

A list of all buffers and solutions used in this study is provided in Table 2.1

2.1.9 Oligonucleotides

A list of all oligonucleotides and their manufacturers is included in Table 2.2

Table 2.1 Buffers and other solutions used in this thesis

Solution	Composition
Acidic Stripping buffer	100 mM acetic acid, 150 mM NaCl, pH 2.7
BES buffer	N,N-bis[2hydroxyethyl]-2-aminoethane sulfonic acid
BOS buffer	50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% NP-40, 10% glycerol, 10 mM NaF, 2.5 mM $MgCl_2$, 1mM EDTA
GST wash buffer	20 mM HEPES PH 7.5, 120 mM NaCl, 10% glycerol, 0.5% NP-40, 2 mM EDTA
Homogenization buffer	20 mM Tris-Cl, pH7.0, 1mM $MgCl_2$, 4mM NaF
Immunoprecipitation buffer	20 mM Tris-Cl, pH7.5, 150 mM NaCl, 1% NP40, 0.1% sodium deoxycholate
Phosphate-buffered saline (PBS)	137 mM NaCl, 2.7mM KCl, 10 mm phosphate buffer
Protease inhibitor cocktail	0.5mM Na_3VO_4, 0.1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10μg/ml aprotinin and 1μM pepstatin A
SDS-loading buffer	250mM Tris-Cl, 40% glycerol, 8% sodium dodecyl sulfate, 20% b-mercaptoethanol, 2% bromophenol blue
SOC medium	2% bacto typtone, 0.5% yeast extract, 10m $MgCl_2$, 10mM MgSO4, 20 mM glucose
Transfer buffer	48 mM Tris-Cl, 39 mM Glycine, 20% methanol, 0.03% sodium dodecyl sulfate
Transformation buffer	10 mM Pipes, 55mM $MnCl_2$, 15mM $CaCl_2$, 250mM KCl
Triton X-100 lysis buffer	0.4% triton X-100, 140mM NaCl, 50mM Tris-Cl, pH 7.2, 1mM EGTA

Table 2.2 Oligonucleotides used in this thesis

Name	Sequence	Manufacturer
PLC-γ1-WT LEADING	TAG AAT TCT ATG GCG GGC GCC GCG TCC CC	Gibco
PLC-γ1-WT LAGGING	GAT GGA TCC GAG GCG GTT GTC TCC ATT GAC	Gibco
PLC-γ1-ΔPH LEADING	GAT AAG CTT GAG GAT ACA TTG CAG GCA CC	Sigma
PLC-γ1-ΔPH LAGGING	GAT AAG CTT AGT CAT GAC GGT GCC CAC CTC	Sigma
PLC-γ1-ΔSH3 LEADING	GAT TCT AGA GTC AAC CCC GTG GCC CTG GAG	Sigma
PLC-γ1-ΔSH3 LAGGING	GAT TCT AGA AGT TGG CAT AGG GTT TGC CTC	Sigma
PLC-γ1-ΔNSH2 LEADING	GTC GAC TGT AAT GAG TTT GAG ATG C	Sigma
PLC-γ1-ΔNSH2 LAGGING	GTC GAC CTT CTC ATT GGA GTG	Sigma
PLC-γ1-ΔCSH2 LEADING	GGT ACC TAC GCA AGA TGA AG	Sigma
PLC-γ1-ΔCSH2 LAGGING	GGT ACC GCT CTC GTG GGC GTT G	Sigma
PLC-γ1-PH LEADING	GGA TCC TGT TCT ACT CCA AGA	Sigma
PLC-γ1-PH LAGGING	GAA TTC CAT CAG CCA AGT TAA GC	Sigma
PLC-γ1-PH PM LEADING	C ACG TGG AGC AAG GAC GAC GAC GAGATC GAG GGG	Sigma
PLC-γ1-PH PM LAGGING	CCC CTC GAT CTC GTC GTC GTC CTT GCT CCA CGT G	Sigma
PLC-γ1-SH3 PM LEADING	G CAG CTG TGG TTC CTA TCA AAC TAC GTG G	Sigma
PLC-γ1-SH3 PM LAGGING	C CAG GTA GTT TGA TAG	Sigma

	GAA CCA CAG CTG C	
PLC-γ1-NSH2 PM LEADING	CC TTC CTC GTG GCA GAG AGT GAG ACC	Sigma
PLC-γ1-NSH2 PM LAGGING	GGT CTC ACT CTC TGC CAC GAG GAA GG	Sigma
PLC-γ1-CSH2 PM LEADING	GCC TTC CTG GTG GCG AAG CGG AAT GAA C	Sigma
PLC-γ1-CSH2 PM LAGGING	G TTC ATT CCG CTT CGC CAC CAG GAA GGC	Sigma
PLC-γ1-S1248A LEADING	GCC CGG GAA GGC GCC TTT GAA TCC CGC TA	Qiagen
PLC-γ1-S1248A LAGGING	TAG CGG GAT TCA AAG GCG CCT TCC CGG GC	Qiagen
PLC-γ1-Y771F LEADING	GCT GAG CCT GAC TTC GGG GCC CTG TAT G	Qiagen
PLC-γ1-Y771F LAGGING	CAT ACA GGG CCC CGA AGT CAG GCT CAG C	Qiagen
PLC-γ1-Y783F LEADING	CGC AAC CCT GGC TTC TTT GTA GAG GCA AAC C	Qiagen
PLC-γ1-Y783F LAGGING	GGT TTG CCT CTA CAA AGA AGC CAG GGT TGC G	Qiagen
PLC-γ1-Y1253F LEADING	CTT TGA ATC CCG CTT CCA GCA GCC GTT TG	Qiagen
PLC-γ1-Y1253F LAGGING	CAA ACG GCT GCT GGA AGC GGG ATT CAA AG	Qiagen
GFP C TEMINUS REVERSE	CAT TTT ATG TTT CAG GTT CAG G	Qiagen
AKT-P424A-LEADING	AAG CTC AGC CCA GCC TTC AAG GCC CAG GTC ACG TCG	Qiagen
AKT-P424A-LAGGING	CGA CGT GAC CTG GGG CCT TGA AGG CTG GGC TGA GC	Qiagen
AKT-P467A-LEADING	AGC GAG CGC AGG GCC CAC TTC GCC CAG TTC TCC TAC	Qiagen

AKT-P467A-LAGGING	GTA GGA GAA CTG GGC GAA GTG GGC CCT GCG CTC GC	Qiagen
AKT-T308A-LEADING	GCC ACC ATC AAG GCC TTT TGC GGC AC	Qiagen
AKT-T308A-LAGGING	GTG CCG CAA AAG GCC CTT CAT GGT GGC	Qiagen
AKT-S473A-LEADING	CTT CCC CCA GTT CGC CTA CTC GGC CAG C	Qiagen
AKT-S473A-LAGGING	GCT GGC CGA GTA GGC GAA CTG GGG GAA G	Qiagen

2.2 Methods

2.2.1 Cell culture

The following cell lines were used in this thesis: Madin-Darby Canine Kidney Epithelial (MDCK) cells (ATCC CCL-34); BT-20 cells (ATCC HTB-19); COS-7 cells (gift of Dr. Tom Hobman, University of Alberta); 293T cells (ATCC CRL-11268); HepG cells (human hepatocellular carcinoma) stably transfected with PDGF Receptor β and F442 cells (Obtained from Dr. David Brindly, University of Alberta). All cells were grown at 37°C in Dulbecco's modified Eagle's medium containing 10% FBS, penicillin, and streptomycin (100U/ml) and were maintained in a 5% CO_2 atmosphere. For HepG and BT-20 cells, non-essential amino acids (Invitrogen Co., Grand Island, NY) were added to a final concentration of 100 μM. For live cell imaging and wound healing assay, COS-7 cells expressing GFP construct were grown in CO_2 independent DMEM (Gibco catalogue number 18045-088).

2.2.2 Cell treatment

To specifically activate EGFR, COS-7, MDCK and BT-20 cells were serum starved for 24 h, and EGF was added to a final concentration of 100 ng/ml. To specifically activate EGFR after its endocytosis into endosomes, MDCK and BT-20 cells were pretreated with 0.5 μM AG1478 for 15 min, and then monensin and EGF were added to a final concentration of 100 μM and 100ng/ml, respectively. After 30 min treatment that is designed as wash for 0 min, the cells were washed with serum-free medium for 3 times,

and then maintained in the serum-free medium which contain 100 μM monensin for the indicated time. Alternatively, MDCK and BT20 cells were pretreated with 0.5 μM AG1478 for 15 min, and then EGF/TR-EGF was added to a final concentration of 100 ng/ml. After a 30 min treatment, the cells were cooled down to 4°C and washed with acidic stripping buffer for 1 min. The cells were then washed with PBS for three times and incubated in serum free medium for the indicated time.

To specifically activate PDGFR, HepG and F442 cells were serum starved for 24 h, and PDGF was added to a final concentration of 10 ng/ml. To specifically activate PDGFR after its endocytosis into endosomes, HepG and F442 cells were serum starved for 24 h. After the cells were pretreated with 0.05 μM AG1296, a specific kinase inhibitor of PDGFR, for 15 min, monensin and PDGF were added to a final concentration of 100 μM and 10 ng/ml, respectively. After a 30 min treatment, the cells were washed with PBS for three times, and then maintained in the serum-free medium for the indicated time.

Two inhibitors, U0126 and PD98059, were used to inhibit MEK activation. U0126 was dissolved in dimethyl sulphoxide (DMSO) at a stock concentration of 10 mM, and PD98059 at 50 mM in DMSO. Prior to stimulation, the cells were cultured overnight in the absence of serum. U0126 was added to the medium to a final concentration of 10 μM and PD98059 to a final concentration of 50 μM for 2 hours. For the depletion of Raf, 10-μM Radicicol was added to the medium and the cells were cultured for a further period of 40 h (Soga et al., 1998; Soga et al., 1998). To inhibit PI3K, 100 nM

wortmannin was added to the medium for 30 min and then incubated with EGF in the continuous presence of wortmannin (100 nM). To inhibit PLC-γ1, the cells were treated with U73122 (10 μM) for 30 min and then stimulated with EGF in the continuous presence of U73122 (10 μM). To inhibit PKA activation, cells were pretreated with H89 (10 μM) for 30 min or KT5720 (500 nM) for 30 min, and then stimulated with EGF in the continuous presence of inhibitors.

2.2.3 Plasmids construction

2.2.3.1 Construction of GFP tagged Rab5

Rab5 was cut from pcDNA3.1-Rab5 (Chen and Wang, 2001) and inserted into the pEYFP-N1 vector (ClonTech, CA). The constructed pEYFP-Rab5 was then used to transiently transfect BT20 cells by calcium phosphate precipitation.

2.2.3.2 Generation of wild-type construct and different mutants of PLC-γ1

As shown in Figure 5.2A, to construct YFP-tagged wild-type PLC-γ1 (YFP-PLC-γ1), PLC-γ1 cDNA was amplified by PCR and then sub-cloned into the pEYFP-C1 vector. To construct the mutant PLC-γ1 with deletion of the PH domain (ΔPH-PLC-γ1), the array before PH domain (encoding residues 1-36) and the array after PH domain (encoding residues 142-1290) were amplified by PCR and then ligated. The ligated DNA sequence was subcloned into the pEYFP-C1 vector using EcoRI and BamHI sites. To construct the mutant PLC-γ1 with deletion of the SH3 domain (ΔSH3-PLC-γ1), the array before SH3 domain (encoding residues 1-791) and the array after SH3 domain

(encoding residues 850-1290) were amplified by PCR and then ligated. The ligated DNA sequence was subcloned into the pEYFP-C1 vector using EcoRI and BamHI sites. To construct the mutant PLC-γ1 with deletion of the NSH2 domain (ΔNSH2-PLC-γ1), the array before N-SH2 domain (encoding residues 1-549) and the array after N-SH2 domain (encoding residues 646-1290) were amplified by PCR and then ligated. The ligated DNA sequences were then subcloned into the pEYFP-C1 vector using EcoRI and BamHI sites. To construct the mutant PLC-γ1 with deletion of the CSH2 domain (ΔCSH2-PLC-γ1), the array before N-SH2 domain (encoding residues 1-665) and the array after N-SH2 domain (encoding residues 747-1290) were amplified by PCR and then ligated. The Ligated DNA sequences were then subcloned into the pEYFP-C1 vector using EcoRI and BamHI sites .

All of the mutants with point mutations were created with the QuikChange multiple site-directed mutagenesis kit (Stratagene) with YFP tagged wild type PLC-γ1 (PLC-γ1) as template. These mutants include a YFP-tagged mutant PLC-γ1 with mutation of Y771 to phenylalanine (termed Y771F), a YFP-tagged mutant PLC-γ1 with mutation of Y783 to phenylalanine (termed Y783F), a YFP tagged mutant PLC-γ1 with mutation of both Y771 and Y783 to phenylalanine (termed Y771F/Y783F), a YFP-tagged mutant PLC-γ1 with mutation of Serine 1248 to Alanine (termed S1248A)

Several glutathione-S-transferase (GST) fusion proteins including GST-N-SH2, GST-C-SH2, GST-SH3, and GST-NC-SH2-SH3 were previously made (Wang et al., 1998). To generate the GST-N-PH, the N-PH domain of PLC-γ1 (encoding residues 37-141) was amplified by PCR and subcloned into the pGEX-3X vector.

2.2.3.3 Generation of the constructs for wild-type and various mutants of Akt

GFP-Akt was a gift from Dr. Julian Downward (Cancer Research UK London Research Institute, UK). All of the mutants with point mutations were created with the QuikChange multiple site-directed mutagenesis kit (Stratagene) with GFP-tagged wild type Akt as template. These mutants a GFP-tagged mutant Akt with mutation of P424 to alanine (P424A), a GFP-tagged mutant Akt with mutation of P476 to alanine (P467A) and a GFP-tagged mutant PLC-γ1 with mutation of both P424 and P476 to alanine (termed P424A/P467A). Glutathione-agarose beads coupled to GST-Akt were bought from Upstate Biotechnology.

2.2.4 Purification of GST fusion PLC-γ1 domains

To purify N-SH2, C-SH2, SH3 and SH2-SH2-SH3 domains of PLC-γ1, the pGEX-3X plasmid containing the wild type N-SH2, C-SH2, SH3 and SH2-SH2-SH3 domains of PLC-γ1 was transformed into *Escherichia coli* DH5α. Cells were grown to an OD_{600} of 0.3-0.4, and induced with 1mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3h at 37°C. After pelleting, cells were lysed by sonication in 50 mM Tris, pH 8.0, 100mM NaCl, 10% glycerol, 1 mM dithiothreitol (DTT), containing protease inhibitors cocktail. Triton X-100 was added to 1% in the lysate and particulates were removed by centrifugation for 10 min at 10000 rpm in a JA-17 (Beckman) rotor. The clarified lysate was incubated with glutathione-agarose beads (Sigma) for 1 h at 4°C, washed three times with ice-cold GST wash buffer containing 1mM DTT plus protease inhibitors, and protein was stored at 4°C bound to the glutathione-agarose beads.

To purify PH domain of PLC-γ1, *Escherichia coli* BL-21, a strain defective in OmpT and Lon protease production, which can express the fusion protein in a more soluble and intact form, were used for transformation. Cells were grown to an OD_{600} of 0.3-0.4, and induced with 0.1 mM IPTG for 8-10 h at 22°C. The later steps were the same as for the other GST-mutants.

2.2.5 GST fusion protein pull-down experiment:

Cell lysates were made as described previously (Wang et al., 1998). Briefly, BT-20, MDCK, COS-7, HepG or F442 cells which had been treated as required were lysed and scraped into 0.5 ml of BOS buffer with protease inhibitors, and then spun at 21000 g at 4°C for 30 min. Supernatants were used in the binding assay. GST fusion proteins of PLC-γ1, pre-coupled to glutathione-agarose-beads in BOS buffer, were added and the lysates were incubated at 4°C for 1 h. Beads were collected by centrifugation, washed three times with BOS buffer, and then subjected to immunoblotting.

2.2.6 Subcellular fractionation

Isolation of plasma membrane (PM), endosomal (EN), and cytosolic (CY) fractions was carried out by a method modified from our previous publication (Wang et al., 1999). For each experimental treatment, four 15-cm-diameter plates of MDCK cells, or eight 15 cm diameter plates of BT-20 cells or F442 cells, or five 15 cm diameter plates of HepG cells were used. Subconfluent cells were serum starved for 24 h prior to treatment. Followed the specific treatment, all of the following procedures were performed at 4°C. For each treatment, cell monolayers from four plates were scraped into 3-4 ml of

homogenization buffer with 0.25M sucrose and protease inhibitor cocktail, and homogenized with a glass Potter-type homogenizer. The homogenates were centrifuged at 200 x g for 5 min to remove cell debris and nuclei (pellet 1 [p1]). The postnuclear supernatant (S1) was then centrifuged at 1500 x g for 10 min to yield a supernatant (S2), which was used to isolate the EN and CY fractions, and a pellet (P2), which was used to isolate the PM fraction. Next, P2 was resuspended in homogenization buffer (0.25 M sucrose), overlaid upon an equal volume of 1.42 M sucrose buffer and centrifuged at 82000 x g for 1 h. The pellicule at the 0.25-1.42 M interface was collected, sucrose concentration adjusted to 0.40 M, and then centrifuged at 20, 000 x g for 30 min to obtain the solid phase PM fraction. The S2 fraction was centrifuged at 200, 000 x g for 30 min to yield the soluble CY fraction and a microsomal pellet. This pellet was resuspended in 0.25 M sucrose buffer and overlaid upon a discontinuous sucrose gradient containing equal volumes of homogenization buffer at 1.00 and 1.15 M sucrose. The resuspension was centrifuged at 200, 000 x g for 1.5 h. To obtain the purified EN fraction, the pellicule at the 0.25-1.00 M interface was collected, sucrose adjusted to 0.25 M, and then centrifuged at 200000 x g for 30 min. Both PM and EN fractions were solubilized overnight using Mammalian Protein Extraction Reagent (M-PER, Pierce Chemical Co., IL) prior to electrophoresis. Due to low yields, fractions from multiple experiments were pooled for subsequent analysis on SDS-PAGE.

The crude Subcellular fractionation experiments were carried out as described previously (Wang et al., 1998). Briefly, following treatment, COS-7 cells were scraped

into homogenization buffer and homogenized. The homogenates were centrifuged at 280 *g* for 5 min to remove cell debris and nuclei. The postnuclear supernatant was centrifuged at 100 000 *g* for 1 h to yield a supernatant (S100) which was collected as cytosol (Cyt) and a particulate (P100) fraction which was dissolved in immunoprecipitation buffer and collected as total membrane (Mem). An immunoprecipitation experiment was performed as described below.

Nuclear extracts were prepared using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Pierce Chemical Co., IL). Briefly, 200 µl of ice-cold CER I buffer was added to 20 µl packed cells. After vigorous vortexing for 15 seconds and incubated on ice for 10 min, 11 µl of ice-cold CER II was added to the tube. The tube was incubated for 1 min and centrifuged for 5 min at 16000 g. The supernatant fraction (cytoplasmic extract) was transferred to a pre-chilled tube, and the insoluble (pellet) fraction was resuspended in 100 µl ice-cold NER for 40 min. After centrifugation of the tube at 16000 g for 10 min, the supernatant fraction (nuclear extract) was transferred to a pre-chilled tube.

2.2.7 Total cell lysates

To obtain total lysates, BT20, MDCK , COS-7, HepG or F442 cells were lysed and homogenized in ice-cold M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, Illinois) with protease inhibitor cocktail. The lysates were centrifuged at 4^0C

for 30 min at 21,000g. Following protein quantification the supernatant was boiled in SDS-loading buffer at 95°C for 5 minutes.

Protein was quantitated using the Bradford protein dye assay, according to the method of Bradford (Bradford, 1976). Absorbance at λ=595nm was measured by a Beckman DU 640 spectrophotometer (Beckman Instrument, Fullerton, CA). Bovine Serum Albumin (BSA) was used as a standard.

2.2.8 Gel electrophoresis and immunoblotting

Aliquots of protein from each sample were used for SDS-polyacrylamide gel electrophoresis. For the staining of total cell lysates, aliquots containing 20µg of protein from each cell lysate were used. For the detection of immunoprecipitation products, one-tenth of the immunoprecipitate from each lysate was used. To detect each subcellular fraction, aliquots containing one-tenth of the total protein from each fraction were used. Protein samples were separated by electrophoresis through 8%-12% SDS-polyacrylamide gels depending on the size of protein being studied. Prestained protein markers (Sigma) were used for molecular weight standards. Proteins were electrophoretically transferred onto Transblot nitrocellulose membranes (BioRad, Hercules, CA). Transfer was done using a semi-dry blotting apparatus (Model SD transfer cell, BioRad) at 15 mA per minigel for 45 minutes in transfer buffer. Blots were blocked with 3% skim milk in 0.05% Tween-PBS (blocking buffer) for 30 minutes. Membranes were then probed with the respective primary antibody (Table 2.3) in blocking buffer overnight, washed twice with distilled H_2O, incubated with HRP-conjugated IgG secondary antibody (Table 2.4)

for 1 hour, washed twice with distilled H_2O, washed with 0.05% Tween-PBS for 5 minutes, rinsed twice with distilled H_2O and washed with TBS buffer. Secondary antibodies were detected by enhanced chemiluminescence, with SuperSignal ECL Western Blotting Detection Reagents (Pierce Chemical) and exposure to X-ray film (Fuji Photo Film Co., Tokyo, Japan) Exposed film was visualized with a digital imaging system (Alpha Innotech Corporation). All antibodies used are listed in Table 2.3 with their respective dilutions.

2.2.9 Fluorescence Microscopy

Indirect immunofluorescence was carried out as described previously (Wang et al., 1996). Briefly, cells were grown on glass coverslips and serum starved for 24 h. After treatment, the cells were fixed by methanol and permeabilized with 0.2% Triton X-100. Next, the cells were incubated with indicated primary antibodies at room temperature for 1 h followed by fluorescence-labeled secondary antibodies for 1 h. The stained cells were analyzed and photographed with a *Zeiss* Axiovert 200 microscope (Thornwood, NY) and an AttoArc2 HBO 100W light source (Atto Instrument, Rockville, MD). Color photographs were taken with a digital camera by superimposing the monochrome graphs of two channels. A list of antibodies used for immunofluorescence and the concentrations at which they were used is given in Table 2.4.

Observation on living cells transfected with YFP fusion constructs were done by using an inverted *Zeiss* microscope with standard YFP filter. For that purpose, 22 mm cover slips were used and placed upside down in a temperature stable cell chamber. The

Table 2.3 Antibodies and their dilutions used for Western Blotting

Antibody	Dilution	Manufacture
Mouse anti-phospho-serine antibody	1:1000	Sigma
Mouse anti-EEA1	1:1000	BD Pharmingen
Mouse anti-GST antibody	1:1000	Santa Cruz
Mouse anti-lamin IgG	1:1000	American Research Products
Mouse anti-phopho-ERK1/2	1:1000	Santa Cruz
Mouse anti-phospho-PLC-γ1 (Tyr 783)	1:1000	Medicore
Mouse anti-phospho-Tyrosine (py99)	1:1000	Santa Cruz
Mouse anti-PLC-γ1	1:1000	Upstate
Mouse anti-tubulin	1:2000	Santa Cruz
Mouse monoclonal anti-Ras	1:500	Upstate
Rabbit anti-ERK	1:2000	Santa Cruz
Rabbit anti-c-Cbl	1:1000	Santa Cruz
Rabbit anti-EGFR(1005)	1:1000	Santa Cruz
Rabbit anti-GFP	1:5000	clonetech
Rabbit anti-Grb2	1:1000	Santa Cruz
Rabbit anti-PDGFR	1:1000	Santa Cruz
Rabbit anti-phosphoserine (1248) of PLC-γ1	1:1000	Upstate
Rabbit anti-phospho-EGFR (py1068)	1:1000	Santa Cruz
Rabbit anti-phospho-EGFR (py1086)	1:1000	Santa Cruz
Rabbit anti-phospho-EGFR (py1148)	1:1000	Santa Cruz
Rabbit anti-phospho-EGFR (py1173)	1:1000	Santa Cruz
Rabbit anti-phospho-EGFR (py992)	1:1000	Santa Cruz
Rabbit anti-phospho-PLC-γ1 (Tyr 783)	1:1000	Santa Cruz
Rabbit anti-phospho-PDGFR	1:1000	Santa Cruz
Rabbit anti-PLC-γ1	1:500	Santa Cruz
Rabbit anti-Raf	1:1000	Santa Cruz
Rabbit anti-SHC antibody	1:1000	BD Transduction laboratory
Rabbit polyclonal anti phospho-Akt (ser 473)	1:1000	Cell signaling
Rabbit polyclonal anti-p85	1:1000	Upstate
Rabbit polyclonal anti-phospho-c-Jun (ser 653)	1:1000	Cell signaling
Horseradish peroxidase(HRP)- conjugated anti-rabbit	1:2000	Bio-Rad
Horseradish peroxidase(HRP)- conjugated anti-mouse	1:2000	Bio-Rad
Horseradish peroxidase(HRP)- conjugated anti-sheep	1:2000	Bio-Rad
Horseradish peroxidase(HRP)- conjugated anti-goat	1:2000	Bio-Rad

Table 2.4 Antibodies and their dilutions used for Immunofluorescence

Antibody	Dilution	Manufactures
Goat anti-PLC-γ1	1:50	Santa Cruz
Mouse anti-EEA1	1:100	BD Pharmingen
Mouse anti-phospho-EGFR	1:100	Upstate
Mouse anti-phospho-PLC-γ1 (Tyr 783)	1:100	Medicore
Mouse anti-phospho-Tyrosine (py99)	1:100	Santa Cruz
Mouse anti-PLC-γ1	1:100	Upstate
Mouse anti-tubulin	1:100	Santa Cruz
Rabbit anti-EGFR(1005)	1:100	Santa Cruz
Rabbit anti-GFP	1:100	Clonetech
Rabbit anti-PDGFR	1:100	Santa Cruz
Rabbit anti-phospho-PLC-γ1 (Tyr 783)	1:100	Santa Cruz
Rabbit anti-phospho-PDGFR	1:100	Santa Cruz
Rabbit anti-PLC-γ1	1:50	Santa Cruz
Rabbit polyclonal anti phospho-Akt (ser 473)	1:100	Cell signaling
Sheep anti-EGFR	1:100	Upstate
Rhodamin-conjugated anti-goat	1:200	Jackson ImmunoResearch
Rhodamin-conjugated anti-mouse	1:200	Jackson ImmunoResearch
Rhodamin-conjugated anti-rabbit	1:200	Jackson ImmunoResearch
Rhodamin-conjugated anti-sheep	1:200	Jackson ImmunoResearch

images were recorded in 1 min intervals. The Video was made using Adobe Premiere 6.0 software (San Jose, CA)

2.2.10 Immunoprecipitation

Immunoprecipitation experiments were carried out as described previously (Wang et al., 1996). The cells were lysed with immunoprecipitation buffer with protease inhibitor cocktail overnight at 4°C. The cell lysates were then centrifuged at 21,000 *g* for 30 min to remove insolubilized debris. The supernatants, containing 1 mg of total proteins were incubated with specific beads for 2 hours at 4°C. After centrifugation at 500 g for 5 min, the supernatants were then incubated with 1 μg of specific IP antibody for 2 h with gentle mixing by inverting. After that, goat anti-mouse IgG conjugated with agarose (Sigma) which can bind mouse IP antibodies or Protein A sepharose 6MB (Pharmacia) which can bind rabbit IP antibodies was added to solution and incubated for 2 h with agitation. Finally, both the beads and the nonprecipitated supernatant were collected by centrifugation. The beads were washed twice with immunoprecipitation buffer, and then resuspended in 1X-loading buffer. The sample was boiled for 5 min and used for western blot. For the control experiments, normal mouse or rabbit IgG (Sigma) was used. A list of antibodies used for immunoprecipitation and their concentrations is given in Table 2.5.

Table 2.5 Antibodies and their concentrations used for immunoprecipitation.

Antibody	Concentration (μg/ml)	Manufactures
Mouse anti-EGFR antibody (Ab1)	1000	Sigma
Mouse anti-PLC-γ1	1000	Upstate
Rabbit anti-EGFR(1005)	200	Santa Cruz
Rabbit anti-GFP	1000	Clonetech
Rabbit anti-PDGFR	200	Santa Cruz
Rabbit anti-PLC-γ1	200	Santa Cruz
Rabbit anti-SHC antibody	1000	BD Transduction laboratory

2.2.11 Ras activation assay

pGEX-3X-RBD encoding (Ras binding domain of Raf, amino acid 51-131) was a gift from Dr. Jim Stone's laboratory (University of Alberta). The bacteria were grown overnight until the OD value is in the range of 0.6-1. Then the bacteria were induced with IPTG to 1 mM final and the bacteria were grown for another 2 hours at 37^{0}C. Resuspend each pellet in 14 ml cold PBSDP (PBS, 1mM DTT and 1/100 pefabloc), followed by French press 4 times, and then Triton-X-100 was added to a final concentration of 1%. The lysate was divided and stored at -80^{0}C. The desired amount of this crude GST-RBD was thawed and incubated with glutathione-agarose beads on ice for 60 min. The beads were isolated by centrifugation and washed 2 times GST wash buffer and 2 times with BOS buffer. BT-20 cells which had been treated as required were lysed and scraped in 0.5 ml BOS buffer, and then spun at 21,000 g and 4^{0}C for 30 min. GST-RBD, precoupled to glutathione-agarose-beads in BOS buffer, was added and the lysates were incubated at 4^{0}C for 1 hour, beads were collected by centrifugation, washed 3 times with BOS buffer, added 100 µl 1 X sample buffer, then boiled for 5min. The protein samples were separated on a 10% SDS PAGE, and transferred to nitrocellulose membrane by western blot. Ras was detected using the monoclonal anti-Ras antibody, followed by HRP-anti-mouse antibody, ECL (Pierce Chemical, Rockford, IL) and light detection with Fuji Super RX Film (Tokyo, Japan).

2.2.12 DNA synthesis assay

DNA synthesis was assayed by bromodeoxyuridine (BrdU) incorporation. Cells (BT20, MDCK, HepG and F442) were plated at 10,000 cells per glass coverslip and serum starved by incubation in serum free medium for 24 h. DNA synthesis was assayed by bromodeoxyuridine (BrdU) incorporation in the presence of 25 μM BrdU. For discontinuous treatment, BrdU was added back after each subsequent pulse or chase. After 16-18 hours, cells were washed with PBS and fixed with methanol (-20°C) for 5 minutes. Following the denaturation of DNA with 2 M HCl for 30 min at room temperature, cells were incubated with mouse anti-BrdU antibody for one hour before addition of FITC-conjugated anti-mouse IgG for (for detection of BrdU) and 50 μg/ml propidium iodide (to stain for total DNA). The stained cells were analyzed and photographed with a Zeiss Axiovert 200 microscope (Thornwood, NY) and an AttoArc2 HBO 100 W light source (Atto Instruments, Rockville, MD). Cell nuclei were visualized in the red and green channels and digital images quantitated for BrdU incorporation. Percent DNA synthesis was calculated as the number of BrdU positive cells/total number of cells analyzed x 100. For each experimental treatment, a minimum of 500 cells was counted.

2.2.13 TUNEL assay

MDCK, BT-20, HepG or F442 cells (10,000 per coverslip) were serum starved for three days to initiate a significant level of apoptosis. Cells were then treated (or not) as described in the DNA Synthesis Assay section, and incubated 20 h with medium alone or

medium containing GF before fixation. As a background control, untreated cells growing in 10% FBS were also assayed to determine the basal apoptotic ratio. Apoptosis was assayed by TdT-mediated dUTP Nick-End Labeling (TUNEL) assay, using an apoptosis detection system kit (Promega) according to the manufacturer's instructions. Briefly, cells were washed, fixed in -20°C methanol for 5 min and then permeabilized with 0.5% Triton X-100 for 10 min. Coverslips were set upon TdT incubation buffer at 37°C for 1 h, and then counter stained with 0.5μg/ml propidium iodide. Cells were visualized in the green channel to morphologically quantitate apoptotic nuclei, and in the red channel to quantitate all nuclei. The percentage of apoptotic cells was calculated as the number of apoptotic nuclei/total nuclei analyzed x 100. For each experimental treatment, a minimum of 250 cells was counted.

2.2.14 Wound healing assay

COS-7 cells were grown in a 24-well plate to 40-60% confluence and transfected with different GFP tagged PLC-γ1 constructs. After 24 to 48 hours, the cells reached 100% confluence and were "wounded" by scraping across the with a glass pipette. A nearby reference point was created by a needle. The plate was washed once and replaced with appropriate medium. The cells were observed under fluorescence microscope to ensure that enough cells in the leading edge of the wound were positively transfected. Images were acquired with both phase contrast and fluorescence images every 2 hours by matching to the reference point until the wound had completely closed. The rate of

migration of the transfected cells was calculated by measuring the distance the cells had traveled toward the center of the wound in 8 hours. The wound healing assay was performed over a 8 hour period which limits the effect of DNA synthesis. At least 8-10 randomly chosen areas from 3-4 separate experiments were quantified, and an individual photograph was chosen as the example.

2.2.15 *In Vitro* Binding Assay

Akt was purified from GST-Akt by thrombin cleavage. Thrombin was dissolved into 1 X PBS to make a stock concentration at 200 U/ml. For each milliliter of packed beads, 50 µl of thrombin solution and 950 µl of 1X PBS were added into the suspension and mixed at room temperature for 2–16 h. After centrifugation at 500 *g* for 5 min to pellet the beads, the supernatant containing the Akt was collected for the in vitro GST pull-down experiments. For the in vitro binding assay, GST and GST-PLC-γ1SH3 beads were incubated with purified Akt for 30 min. After washing three times, the final precipitates were blotted with anti-GST and anti-Akt antibodies.

Chapter 3

Endosomal signaling of EGF receptor stimulates signal transduction pathways leading to cell survival

The majority of the information presented in this chapeter has been published in Wang, Y., S. Pennock, X. Chen, and Z. Wang. 2002 Endosomal signaling of epidermal growth factor receptor stimulates signal transduction pathways leading to cell survival. *Mol. Cell. Biol.* 22 : 7279-7290. It has been adapted into this Thesis with permission.

3.1 Overview

As introduced in Chapter 1, accumulated evidence suggests that the internalized EGF-EGFR complex may maintain its ability to generate cell signaling from endosomes. Internalized EGFR is autophosphorylated and catalytically active (Cohen and Fava, 1985; Kay et al., 1986; Lai et al., 1989). Various signaling molecules that regulate Ras activity, including Grb2, SHC, GAP and Cbl, are co-internalized with EGFR into endosomes and remain associated with the receptor in endosomes (Clague and Urbe, 2001; Di Guglielmo et al., 1994; Fukazawa et al., 1996; Levkowitz et al., 1998; Wang and Moran, 1996). Recently, other reports have confirmed the interaction between EGFR and various signaling proteins in endosomes (Burke et al., 2001; Haugh et al., 1999; Kuruvilla et al., 2000; Miller and Lefkowitz, 2001; Slepnev and De Camilli, 2000). More evidence supporting endosomal signaling comes from endocytosis blocking experiments. This endocytosis-inhibition approach has its limitations. While inhibition of EGFR endocytosis eliminates endosomal signaling, the retention of EGFR at the cell surface also enhances signaling from the plasma membrane. Thus, it is difficult to determine whether the observed effects are due to the lack of endosomal signaling or due to prolonged plasma membrane signaling. Blocking EGFR endocytosis by mutant dynamin or β-arrestin affects all endocytic events mediated by these factors. Thus, it is difficult to determine whether the observed effects are due to the inhibition of EGFR endosomal signaling or due to a broad inhibition of endocytosis. Moreover, this approach is not suitable for studying the dynamics of endosomal signaling. So far, none of the current

approaches offered a means to activate receptors inside a cell without initial activation at the cell surface (Wiley and Burke, 2001)

Here I report the establishment of a system that allows the specific activation of endosome-associated EGFR without the activation of the plasma membrane-associated EGFR and without the disruption of the overall endocytotic pathway. By using this novel system, I provide original evidence demonstrating that 1) endosomes can serve as platforms for the formation of signaling complexes, 2) endosomal EGFR signaling is sufficient to activate the major signaling pathways leading to cell proliferation and survival, and 3) endosomal EGFR signaling is sufficient to suppress apoptosis induced by serum-withdrawal.

3.2 Establishment of a novel system that specifically activates endosome-associated EGFR

Mutation of sequence responsible for the intrinsic tyrosine kinase activity of EGFR abolishes ligand-induced down-regulation, however, whether this is due to impaired internalization or impaired lysosomal targeting remains unsolved (Chen et al., 1989; Felder et al., 1990; Glenney, Jr. et al., 1988). Some studies have shown that the kinase-deficient mutant EGFR K721A is internalized into endosomes in response to EGF. Unlike wild-type EGFR, however, EGFR K721A recycles back to the plasma membrane rather than traffick to lysosomes for degradation (Felder et al., 1990). Treatment of cells with monensin, which inhibits EGFR recycling, results in the retention of EGFR K721A

in endosomes (Felder et al., 1990).

Based on these observations, cells were treated with AG1478 and monensin to specifically inhibit EGFR kinase activation and recycling respectively to examine if the treatement would result in the internalization of non-activated EGF-EGFR complexes into endosomes in response to EGF stimulation. AG1478 is a highly selective inhibitor for EGFR tyrosine kinase (Haugh et al., 1999). AG1478 competes with ATP binding and reversibly inhibits tyrosine trans-phosphorylation, thereby blocking downstream signaling (Arteaga, 2001). Monensin is a cationic ionophore that exchanges Na^+ for H^+, thereby dissipating transmembrane pH gradients and increasing the pH of endosomes. Although multiple intracellular consequences of monensin treatment might be expected, the most widely reported effect of monensin is to block internalized receptors from recycling back to the cell surface (Basu et al., 1981; Wileman et al., 1984; Stein et al., 1984; Pippig et al., 1995).

BT20 cells were treated with AG1478 for 15 min and then stimulated with EGF in the presence of monensin. Immunofluorescence showed that EGFR completely internalized into endosomes after addition of EGF and monensin for 30 min (Figure 3.1A). There is no overlap between EGFR and pTyr staining, indicating that the endosome-associated EGFR was not phosphorylated (inactive) (Figure 3.1A). For control cells treated only with EGF (termed as "standard EGF stimulation"), EGFR was phosphorylated and internalized into endosomes (Figure 3.1A). To more specifically determine the phosphorylation status of endosome-associated EGFR, BT20 cells were double-stained

with anti-EGFR and anti-phospho-EGFR (p-EGFR) antibodies following the treatment described above. The results of the staining confirmed that treatment of BT20 cells with AG1478, EGF and monensin induced internalization of non-phosphorylated EGFR into endosomes (Figure 3.1B).

To determine whether endosome-associated non-phosphorylated EGFR binds to EGF, BT20 cells were treated with TR-EGF (Texas Red-conjugated EGF) in the presence of AG1478 and monensin. Triple immunofluorescence staining indicated that both TR-EGF and the non-phosphorylated EGFR co-localized at endosomes (Figure 3.1C). For control cells treated only with TR-EGF, EGFR was phosphorylated and co-internalized with TR-EGF into endosomes (Figure 3.1C).

To determine whether endosome-associated non-phosphorylated EGFR could be activated, BT20 cells were washed with medium. The endosome-associated EGFR was significantly activated 30 min following wash (Figure 3.1A-C). The activated EGFR remained co-localized with TR-EGF (Figure 3.1C). Similar results were also obtained with MDCK cells by immunofluorescence (Figure 3.1D), which suggest that this observation is not limited to BT20 cells.

These observations were further confirmed by subcellular fractionation experiments (Figure 3.2). It is well established that EGFR is non-phosphorylated and localized at the plasma membrane without EGF stimulation but becomes phosphorylated and localized in endosomes following EGF stimulation for 30 min. Thus EGFR itself could be used as a

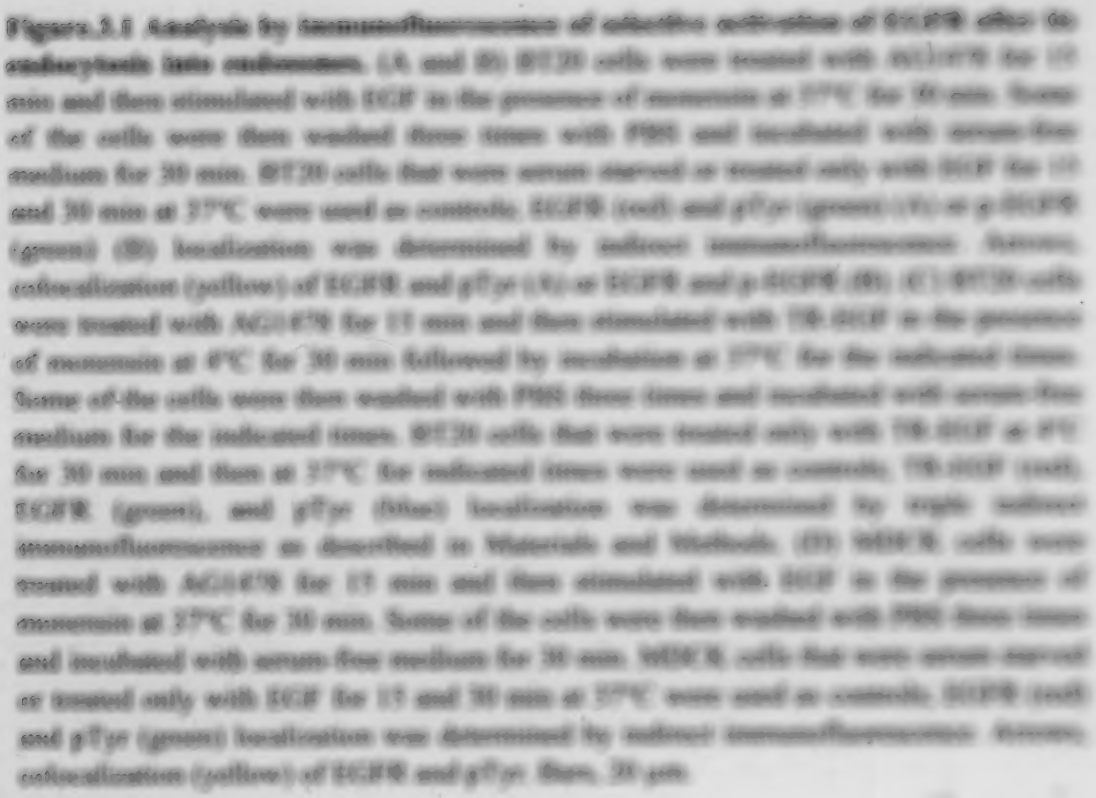
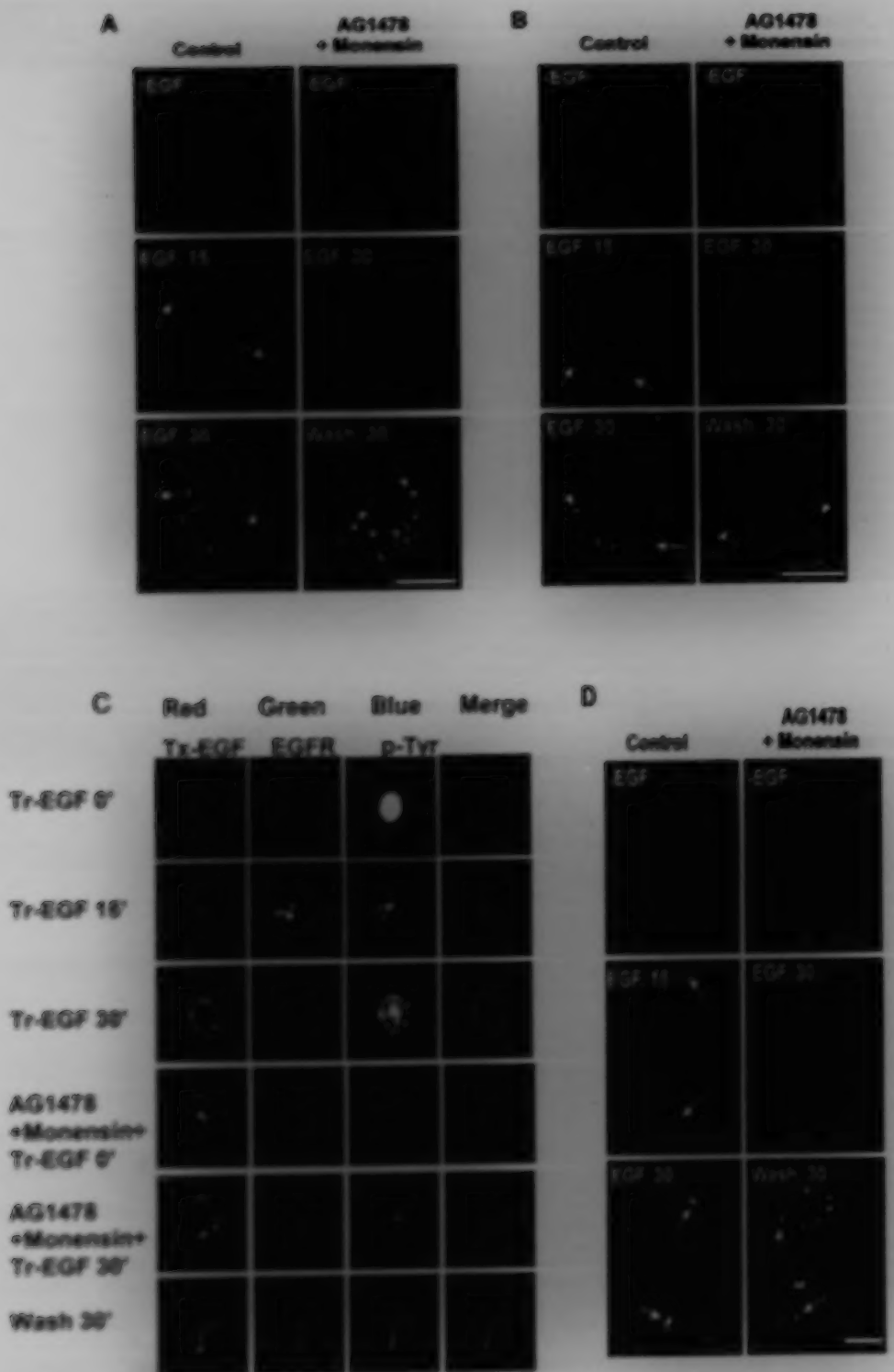


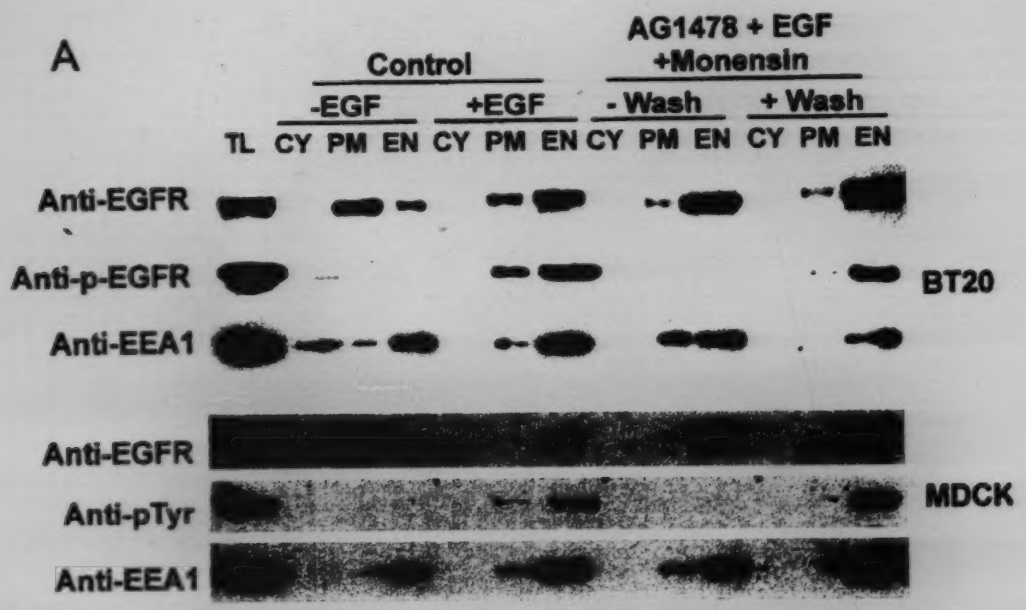
Figure 3.1 Analysis by immunofluorescence of selective activation of EGFR after its endocytosis into endosomes. (A and B) BT20 cells were treated with AG1478 for 15 min and then stimulated with EGF in the presence of monensin at 37°C for 30 min. Some of the cells were then washed three times with PBS and incubated with serum-free medium for 30 min. BT20 cells that were serum starved or treated only with EGF for 15 and 30 min at 37°C were used as controls. EGFR (red) and pTyr (green) (A) or p-EGFR (green) (B) localization was determined by indirect immunofluorescence. Arrows, colocalization (yellow) of EGFR and pTyr (A) or EGFR and p-EGFR (B). (C) BT20 cells were treated with AG1478 for 15 min and then stimulated with TR-EGF in the presence of monensin at 4°C for 30 min followed by incubation at 37°C for the indicated times. Some of the cells were then washed with PBS three times and incubated with serum-free medium for the indicated times. BT20 cells that were treated only with TR-EGF at 4°C for 30 min and then at 37°C for indicated times were used as controls. TR-EGF (red), EGFR (green), and pTyr (blue) localization was determined by triple indirect immunofluorescence as described in Materials and Methods. (D) MDCK cells were treated with AG1478 for 15 min and then stimulated with EGF in the presence of monensin at 37°C for 30 min. Some of the cells were then washed with PBS three times and incubated with serum-free medium for 30 min. MDCK cells that were serum starved or treated only with EGF for 15 and 30 min at 37°C were used as controls. EGFR (red) and pTyr (green) localization was determined by indirect immunofluorescence. Arrows, colocalization (yellow) of EGFR and pTyr. Bars, 20 μm.


A
Control
AG1478
+ Monensin
EGF
EGF 15'
EGF 30'
Wash 30'
B
Control
AG1478
+ Monensin
EGF
EGF 15'
EGF 30'
Wash 30'
C
Red
Tx-EGF
Green
EGFR
Blue
p-Tyr
Merge
Tr-EGF 0'
Tr-EGF 15'
Tr-EGF 30'
AG1478
+Monensin+
Tr-EGF 0'
AG1478
+Monensin+
Tr-EGF 30'
Wash 30'
D
Control
AG1478
+ Monensin
EGF
EGF 15'
EGF 30'
Wash 30'

marker for the plasma membrane and endosomes. Indeed, the results showed that, for both BT20 and MDCK cells, EGFR was primarily in the plasma membrane fraction and not phosphorylated without EGF stimulation, while EGFR was primarily in the endosome fraction and phosphorylated following standard EGF stimulation (Figure 3.2A and Table 3.1). Additionally, early endosome autoantigen 1 (EEA1), a marker for endosomes, is highly enriched in our endosome fractions. Treatment with AG1478, monensin and EGF resulted in the internalization of non-phosphorylated EGFR into endosomes and the endosome-associated EGFR was phosphorylated following washing and incubation in medium (Figure 3.2A and Table 3.1). Washing away of AG1478 and monensin did not result in the increase of plasma membrane-associated EGFR (Figure 3.2A and Table 3.1), which suggests that there is no detectable recycling of EGFR from endosome to the plasma membrane following the removal of monensin. More importantly, no phosphorylated EGFR was detected at the plasma membrane (Figure 3.2A and Table 3.1). A time course of immunofluorescence further showed that no EGFR recycling was detectable following the removal of monensin and there was no detectable EGFR phosphorylation at the plasma membrane (Figure 3.2B). Together, these results suggest that a system has been established in which EGFR in endosomes can be specificially activate without any detectable EGFR activation at the plasma membrane.

Although monensin was used in this system to block the recycling of inactive EGFR back to the plasma membrane, it was washed away prior to the activation of EGFR and thus its non-physiological effects on the endosomal signaling of EGFR are likely to

Figure 3.2 Further analysis of selective activation of endosome-associated EGFR by subcellular fractionation and by the time course of immunofluorescence. (A) Subcellular fractionation. BT20 and MDCK cells were treated with AG1478, EGF, and monensin as described in Materials and Methods. The cells were then subcellularly fractionated into PM, EN, and CY fractions. The subcellular fractions were subjected to immunoblotting with anti-EGFR, anti-pTyr, and anti-EEA1 antibodies. TL, total lysate. (B) Time course by immunofluorescence of the specific activation of endosome-associated EGFR following wash. BT20 cells were treated with AG1478, EGF, and monensin for 30 min and then washed with PBS for the indicated times. EGFR (red) and pTyr (green) localization was determined by indirect immunofluorescence. Arrows, colocalization (yellow) of EGFR and pTyr. Bar, 20 μm.


A
Control
AG1478 + EGF
+Monensin
-EGF
+EGF
- Wash
+ Wash
TL CY PM EN CY PM EN CY PM EN CY PM EN
Anti-EGFR
Anti-p-EGFR
Anti-EEA1
BT20
Anti-EGFR
Anti-pTyr
Anti-EEA1
MDCK

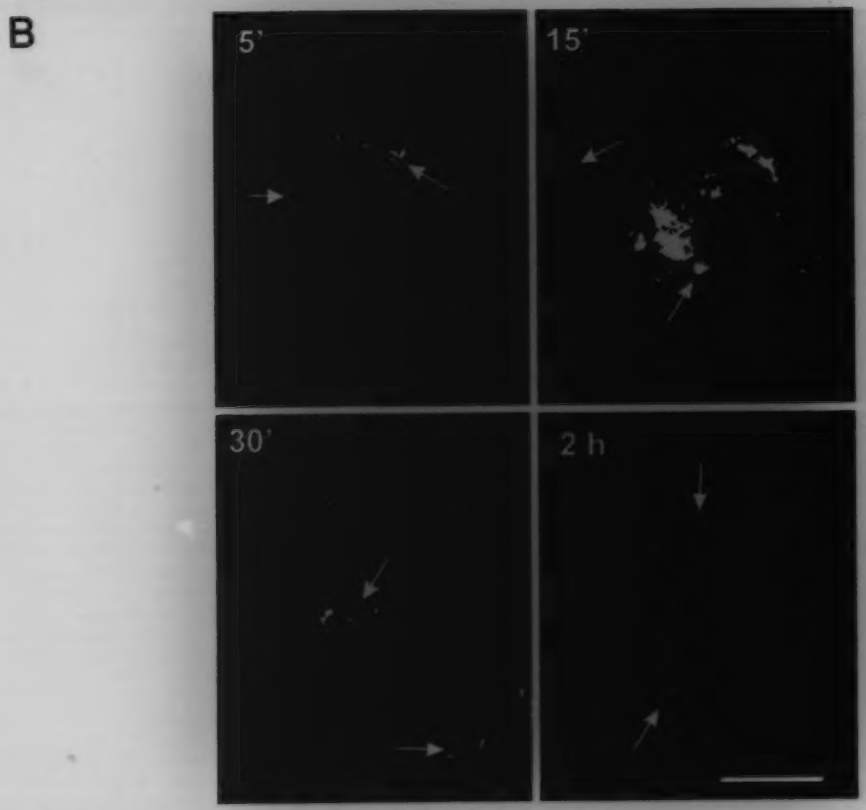

B
5'
15'
30'
2 h

Table 3.1 Analysis by subcellular fractionation of the selective activation of endosome associated EGFR

Cell type and marker	Yield (%) of indicated fraction[a] for:											
	Control cells						Cells treated with AG1478 + EGF + monensin					
	Without EGF			With EGF			Without wash			With wash		
	CY	PM	EN	CY	PM	EN	CY	PM	EN	CY	PM	EN
BT20												
EGFR	0	78	22	0	28	72	0	9	91	0	10	90
p-EGFR	0	0	0	0	27	73	0	0	0	0	2	98
EEA1	29	8	63	3	17	80	2	35	63	4	2	94
MDCK												
EGFR	0	94	5	5	15	80	0	4	95	2	2	96
p-EGFR	0	0	0	0	9	91	0	0	0	0	3	97
EEA1	12	7	81	5	14	81	3	19	78	3	6	91

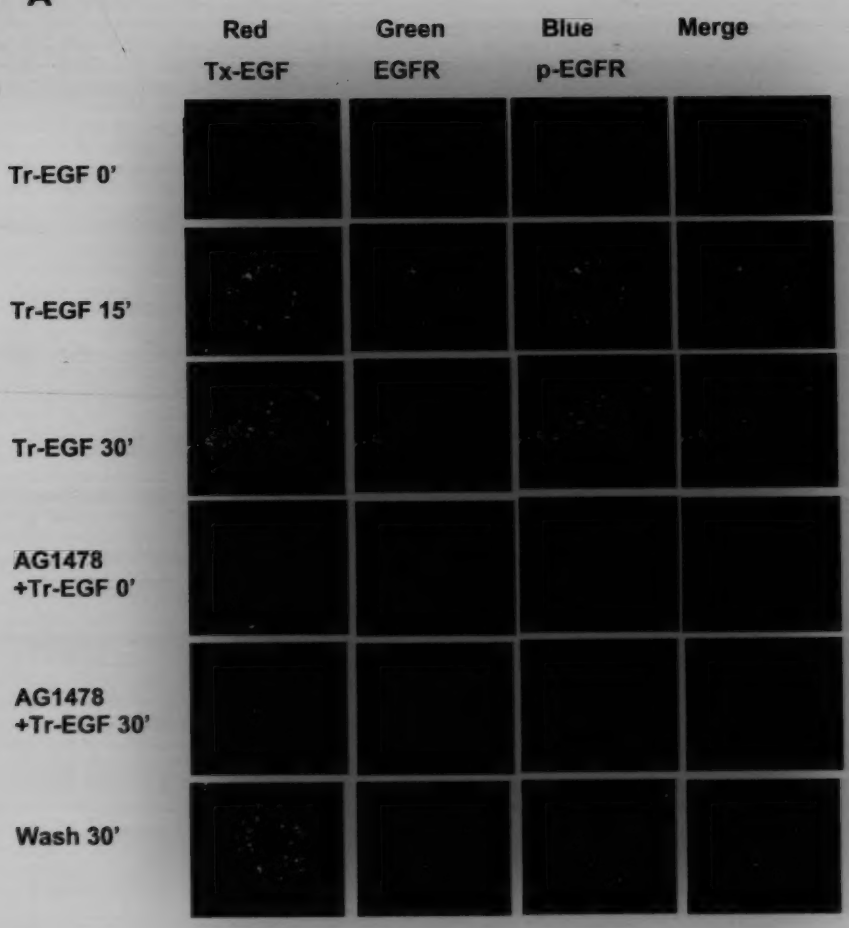
[a] Data are the quantification of the results from Fig. 2A normalized against the total yield of the three fractions. Each value is the percentage of the total of each set of three fractions following normalization.

be minimal.

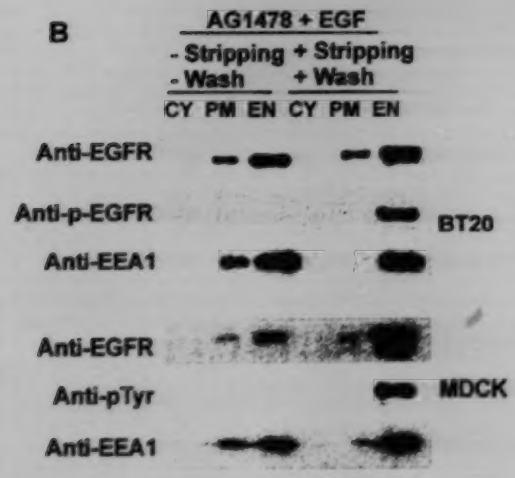
Nevertheless, to completely eliminate any possible non-physiological effects of monensin, I developed another technique to specifically activate endosome-associated EGFR without using monensin. BT20 cells were treated with TR-EGF and AG1478 in the absence of monensin. Interestingly, following incubation at 37°C for 30 min, most TR-EGF and EGFR were localized to endosomes with a small amount of EGFR localized to the plasma membrane (Figure 3.3A). There is no detectable p-EGFR staining, indicating that the endosome-associated EGFR was non-phosphorylated (Figure 3.3A). To dissociate TR-EGF from EGFR at the plasma membrane, cells were cooled down to 4°C and washed with acidic stripping buffer for 1 min. No TR-EGF was detectable at the plasma membrane following stripping. I then activate endosome-associated EGFR by washing the cells with PBS and incubating the cells in serum-free medium at 37°C. At 30 min, the endosome-associated EGFR was significantly activated, but no EGFR at the plasma membrane was phosphorylated (Figure 3.3A). These observations were further confirmed by subcellular fractionation experiments (Figure 3.3B). Treatment with AG1478, monensin and EGF previously had resulted in the internalization of non-phosphorylated EGFR into endosomes and the endosome-associated EGFR was phosphorylated following washing and incubating in medium. No phosphorylated EGFR was detected at the plasma membrane (Figure 3.3B). These results indicate that I am able to specifically activate endosome-associated EGFR without using monensin. With these two techniques, I have established a system by two techniques to specifically activate

Figure 3.3 Selective activation of endosome-associated EGFR without monensin. (A) BT20 cells were treated with AG1478 for 15 min and then stimulated with TR-EGF at 4°C for 30 min, followed by a wash with acidic stripping buffer at 4°C for 1 min. The cells were then washed with PBS three times and incubated with medium at 37°C for the indicated times. BT20 cells were triple fluorescence stained for TR-EGF (red), EGFR (green), and p-EGFR (blue) as described in Materials and Methods. Arrows, colocalization (yellow) of TR-EGF and EGFR; arrowheads, colocalization (light purple) of TR-EGF, EGFR, and p-EGFR. Bar, 20 μm. (B) Subcellular fractionation. BT20 and MDCK cells were treated with AG1478 for 15 min and then stimulated with EGF at 37°C for 30 min, followed by a wash with acidic stripping buffer at 4°C for 1 min. The cells were then washed with PBS three times and incubated with medium at 37°C for the indicated times. The cells were then subcellularly fractionated into the PM, EN, and CY fractions. The subcellular fractions were subjected to immunoblotting with anti-EGFR, anti-pTyr, and anti-EEA1 antibodies as described in Materials and Methods. TL, total lysate.

A


Red
Tx-EGF
Green
EGFR
Blue
p-EGFR
Merge
Tr-EGF 0'
Tr-EGF 15'
Tr-EGF 30'
AG1478
+Tr-EGF 0'
AG1478
+Tr-EGF 30'
Wash 30'

B


AG1478 + EGF
- Stripping
- Wash
+ Stripping
+ Wash
CY PM EN CY PM EN
Anti-EGFR
Anti-p-EGFR
BT20
Anti-EEA1
Anti-EGFR
Anti-pTyr
MDCK
Anti-EEA1

endosome-associated EGFR without any detectable activation of EGFR at the plasma membrane.

3.3 EGFR activity and trafficking in our established system

I next compared EGFR activity and trafficking in our system with that following standard EGF stimulation. I first determined whether treatment with AG1478 and EGF, with or without monensin, resulted in the internalization of EGFR into the same pool of endosomes as that following standard EGF stimulation. It is well established that following EGF stimulation, phosphorylated EGFR is internalized into Rab5-positive endosomes prior to its trafficking to late endosomes and lysosomes (Barbieri et al., 2000; Chen and Wang, 2001; Lanzetti et al., 2000). BT20 cells were transiently transfected with Rab5 (Chen and Wang, 2001). I showed that treatment with AG1478 and EGF, with or without monensin, also results in the internalization of non-phosphorylated EGFR into Rab5-positive endosomes (Figure 3.4A). Following washing and incubating with medium for 30 min to activate the endosome-associated EGFR, EGFR was still mostly localized to Rab5-positive endosomes (Figure 3.4A). In control cells treated with EGF for 30 min, EGFR was localized to Rab5-positive endosomes as expected (Figure 3.4A). These results indicate that treatment with AG1478 and EGF with or without monensin result in the internalization of EGFR into the same pool of endosomes as that following standard EGF stimulation. I then determined phosphorylation and degradation of EGFR. BT20 and MDCK cells were treated with AG1478 and EGF with or without monensin as described above. The pattern of dephosphorylation was very similar to that following standard EGF

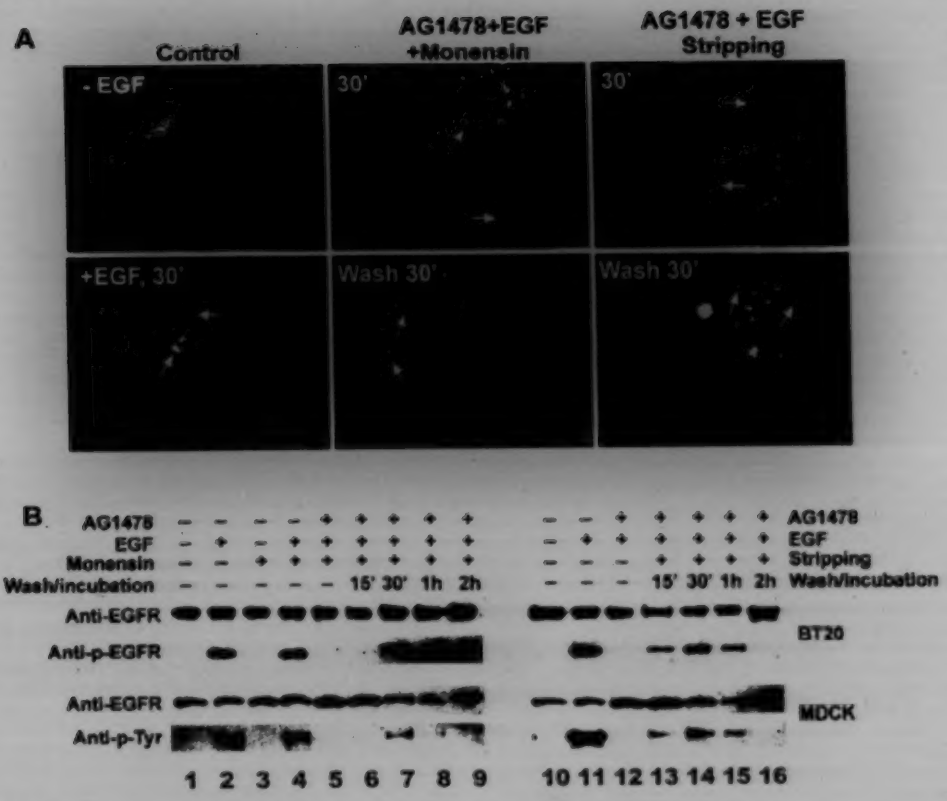


Figure 3.4. Trafficking and activity of EGFR following its selective activation in endosomes. (A) Internalization of inactive EGFR into Rab5-positive endosomes and its subsequent activation. BT20 cells were transfected with wild-type Rab5. Cells were either not treated or treated with EGF for 30 min as controls (left). Some cells were treated with AG1478 for 15 min, followed by addition of EGF and monensin for 30 min without or with washing and incubation (middle). Some cells were treated with AG1478 and EGF for 30 min without washing or followed by washing with acidic stripping buffer for 1 min and incubation with medium for 30 min. EGFR (red) and Rab5 (green) localization was determined by indirect immunofluorescence. Arrows, colocalization (yellow) of EGFR and Rab5. (B) Immunoblot analysis of the trafficking and inactivation of EGFR following its selective activation in the endosome. BT-20 and MDCK cells were serum starved overnight. Lane 1 and 10, cells were not treated with anything; Lane 2 and 11, cells were treated with EGF; Lane 3, cells were treated with monensin; Lane 4, cells were treated with monensine and EGF; Lane 5, cells were treated with AG1478, monensine and EGF; Lane 6-9, cells treated with AG1478, monensine and EGF were washed with different time; Lane 12, cells were treated with AG1478 and EGF; Lane 13-16, cells cells treated with AG1478 and EGF were acidic washed with different time. Cell lysates were subjected to immunoblot analysis with mouse anti-pTyr, anti-p-EGFR, and rabbit anti-EGFR antibodies.

stimulation. These results indicate that EGFR activation, trafficking and deactivation in our system are very similar to that following standard EGF stimulation.

3.4 Recruitment of signaling proteins into endosomes by activation of endosome-associated EGFR

I next used this system to specifically study EGFR-mediated signal transduction from endosomes. Following EGF stimulation, various signaling proteins, including Grb2, SHC, the p85α subunit of PI3K and PLC-γ1, are recruited to the plasma membrane by the interaction between EGFR pTyr sites and the signaling proteins (Pawson, 1995; Yarden and Sliwkowski, 2001). Many of these proteins, including Grb2, SHC and GAP are then co-internalized with activated EGFR and remain associated with the EGFR in endosomes (Clague and Urbe, 2001; Di Guglielmo et al., 1994; Fukazawa et al., 1996; Levkowitz et al., 1998; Wang and Moran, 1996). However, it is unclear whether the direct activation of endosome-associated EGFR is able to recruit these signaling proteins directly into endosomes, or whether the interaction between EGFR and these signaling proteins has to be initiated at the plasma membrane. To answer this question, BT20 and MDCK cells were treated with AG1478 and EGF with or without monensin as described above. The cells were then lysed and immunoprecipitated with anti-EGFR antibody. Immunoblotting of the immunoprecipitates revealed the association of EGFR with various signaling proteins following the activation of endosome-associated EGFR (Figure 3.5). The signaling proteins that are co-immunoprecipitated with EGFR include SHC, Grb2, and the p85α subunit of PI3K. These results indicate that direct activation of

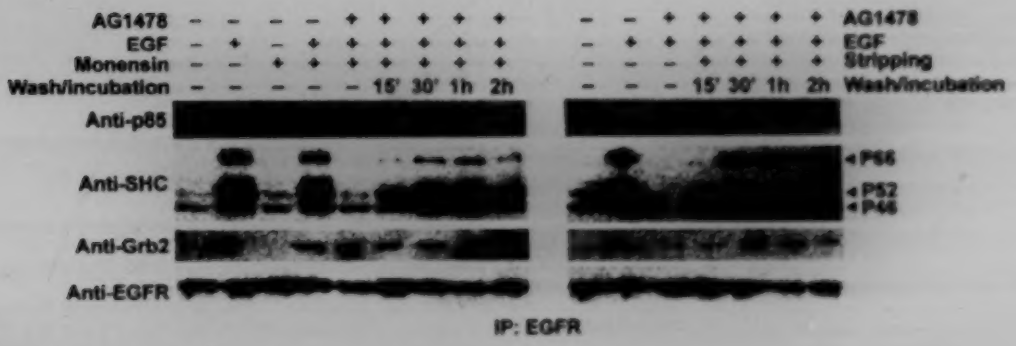


Figure 3.5 Interactions between signaling proteins and endosome-associated EGFR in BT20 cells. (A) BT20 cells were treated with AG1478 and EGF with or without monensin as described in Materials and Methods. The cells were washed and then incubated with serum-free medium for the indicated times. The cells were lysed and immunoprecipitated (IP) with a rabbit anti-EGFR antibody as described in Materials and Methods. Immunoprecipitates were subjected to immunoblot analysis with mouse anti-EGFR, anti-Grb2, anti-SHC, and anti-p85α antibodies. BT20 cells that were serum starved or treated with EGF, monensin, or EGF plus monensin were used as controls.

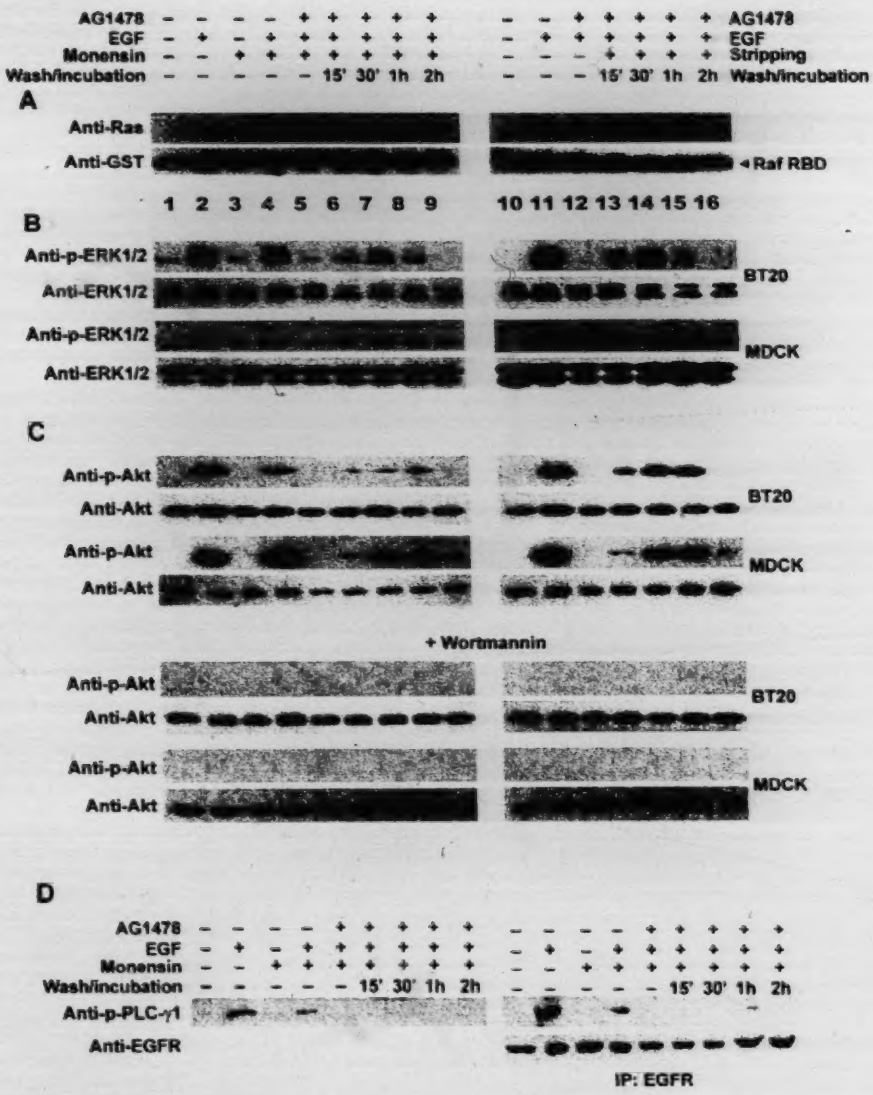
endosome-associated EGFR is sufficient to recruit some signaling proteins into endosomes.

3.5 Stimulation of various signaling pathways by endosomal signaling of EGFR

I further determined whether endosomal signaling of EGFR is sufficient to stimulate various signaling pathways. It is well established that following standard EGF stimulation, several signal transduction pathways are activated including the Ras-ERK pathway that is essential for cell mitogenesis (Marshall, 1996) and the PI3K-Akt pathway that protects against apoptosis (Burgering and Coffer, 1995; Downward, 1998). I showed that activation of endosome-associated EGFR also significantly activated Ras (Figure 3.6A), ERK1/2 (Figure 3.6B) and Akt (Figure 3.6C). Interestingly, while the activation level of endosome-associated EGFR is approximately 50% of that following standard EGF stimulation (Figure 3.4), the activation levels of Ras, ERK1/2 and Akt were also approximately 50% of that following standard EGF stimulation (Figure 3.6). These results suggest that activation of endosome-associated EGFR and the standard activation of EGFR may have similar effects on the activation of Ras-ERK and Akt pathways.

Moreover, the Akt phosphorylation was blocked by the PI3K inhibitor wortmannin, indicating that Akt activation is mediated by PI3K (Figure 3.6C). Together, these results indicate that endosomal signaling of EGFR is sufficient to stimulate signal transduction pathways implicated in cell mitogenesis and survival.

However, activation of endosome-associated EGFR only slightly activated PLC-γ1.


AG1478
EGF
Monensin
Wash/incubation
15' 30' 1h 2h
Stripping
A
Anti-Ras
Anti-GST
Raf RBD
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
B
Anti-p-ERK1/2
Anti-ERK1/2
BT20
MDCK
C
Anti-p-Akt
Anti-Akt
+ Wortmannin
D
Anti-p-PLC-γ1
Anti-EGFR
IP: EGFR
E
120%
100%
80%
60%
40%
20%
0%
EGFR
Ras
ERK1/2
Akt
SF
Standard EGF stimulation
Endosomal stimulation (with monensine)
Endosomal stimulation (acidic wash)

While no phosphorylation of PLC-γ1 could be detected in the total lysates following activation of endosome-associated EGFR (Figure 3.6D), I detected phosphorylation of PLC-γ1 from EGFR immunoprecipitates (Figure 3.6D).

3.6 Support of cell survival by endosomal signaling of EGFR

I finally determined whether pure endosomal signaling of EGFR is sufficient to produce a biological outcome. I examined the effects of pure endosomal signaling of EGFR on serum-withdrawal induced apoptosis by TUNEL assay (Figure 3.7). For BT20 cells cultured in 10% FBS, only 5% of cells are apoptotic; however, serum-withdrawal for 24 h induced 54% apoptosis. For MDCK cells serum-withdrawal for 72 h induced 47% apoptosis, compared with 3% apoptosis in 10% FBS (Figure 3.7). To determine the effects of activation of endosome-associated EGFR on apoptosis induced by serum-withdrawal, BT20 and MDCK cells were stimulated with EGF (20 ng/ml) for 30 min in the presence of AG1478 with or without monensin to allow the internalization of inactive EGFR into endosomes. After the subsequent activation of endosome-associated EGFR by stripping and/or washing and incubating with serum-free medium for 12 h, the percentage of apoptotic cells was significantly reduced to a similar level following standard EGF (20 ng/ml) stimulation for 30 min (Figure 3.7). These results demonstrate that pure endosomal signaling of EGFR is sufficient to produce a biological effect. Moreover, when the cells were pretreated with wortmannin (100 nM) to block PI3K activity, the activation of endosome-associated EGFR was unable to suppress apoptosis induced by serum-withdrawal (Figure 3.7). This indicates that, similar to standard

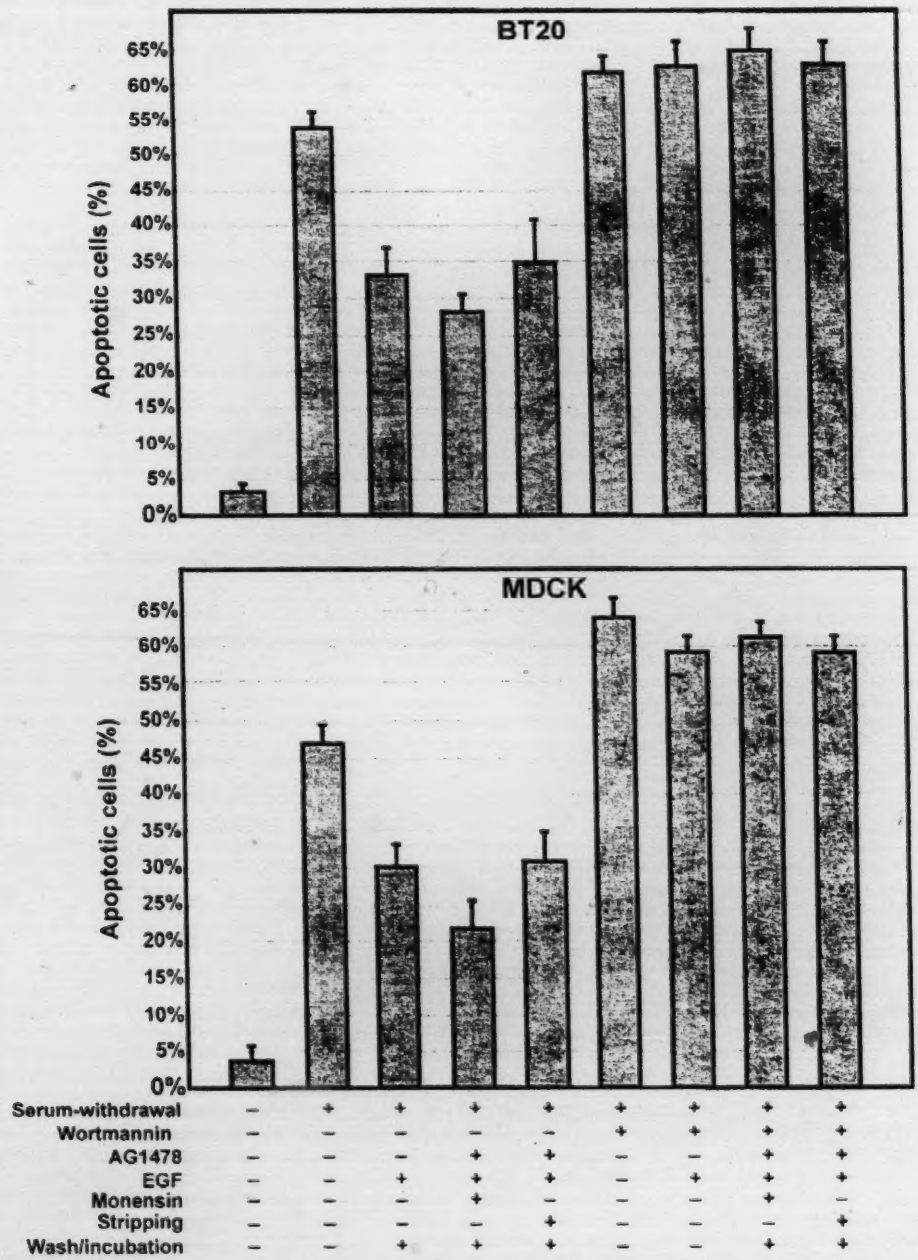


Figure 3.7 Activation of endosome-associated EGFR and the effects on cell survival. BT20 and MDCK cells were incubated in serum-free medium to induce apoptosis. The cells were then treated AG1478 and EGF (20 ng/ml) with or without monensin followed by washing and incubation as described above. To determine the effects of wortmannin, the cells were pretreated with wortmannin (100 nM). The apoptosis was determined by TUNEL assay.

activation of EGFR, activation of endosome-associated EGFR supports cell survival by stimulating the PI3K-Akt pathway.

3.8 Summary

In summary, I have successfully established a system to allow the specific activation of endosome-associated EGFR without the initial activation at the plasma membrane. By using this system, I determined the roles of endosomal EGFR signaling in cell mitogenesis and anti-apoptosis. I provided original evidence demonstrating that endosomes can serve as nucleation sites for the formation of signaling complexes and that endosomal EGFR signaling is sufficient to activate the major signaling pathways leading to cell proliferation and anti-apoptosis. I also demonstrated that pure endosomal EGFR signaling is sufficient to suppress apoptosis induced by serum-withdrawal.

Chapter 4

PDGF receptor-mediated signal transduction from endosomes

The majority of the information presented in this chapeter has been published in Wang, Y., S. Pennock, X. Chen, A. Kazlauskas, and Z. Wang. 2004. PDGF receptor-mediated signal transduction from endosomes. *J. Biol. Chem.*, 279: 8038-8046. It has been adapted into this Thesis with permission.

4.1 Overview

Platelet-derived growth factor (PDGF) is a connective tissue cell mitogen that binds the PDGF receptor (PDGFR) with high affinity (Hart et al., 1988; Heldin and Westermark, 1989). Like other receptor tyrosine kinases (RTKs), the PDGFR possesses an extracellular region (containing five immunoglobulin-like domains), a single transmembrane segment, a juxtamembrane segment, a protein tyrosine kinase domain and a carboxyl-terminal tail (Heldin et al., 1998; Heldin and Westermark, 1999). Binding of PDGF to PDGFR induces the dimerization of PDGFR (Heldin and Westermark, 1989; Bishayee et al., 1989), which results in the activation of its intrinsic tyrosine kinase activity followed by trans-autophosphorylation (Ullrich and Schlessinger, 1990; Kelly et al., 1991). The phosphotyrosine residues recruit signaling proteins containing pTyr-binding domains such as SH2 or PTB domains. Several of these signaling proteins have been identified, and include Src kinase family members (Kypta et al., 1990), phospholipase C-γ1 (Meisenhelder et al., 1989; Wahl et al., 1990), the p85α subunit of phosphatidylinositol 3' kinase (PI3K) (Kazlauskas and Cooper, 1989), GTPase-activating protein (GAP) (Kashishian et al., 1992; Fantl et al., 1992), the phosphotyrosine phosphatase SHP-2 (Lechleider et al., 1993; Kazlauskas, 1993), and adaptor proteins such as Grb2 (Rozakis-Adcock et al., 1992), SHC (Yokote et al., 1994), Grb7 (Stein et al., 1994) and Nck (Nishimura et al., 1993). The formation of receptor-signaling protein complexes then initiates the activation of various signaling pathways including the Ras-Erk pathway, PI3K-Akt pathway, PLC-γ1 pathway and Src pathway. The activation

of these pathways eventually leads to cell proliferation and survival.

While endocytosis has traditionally been considered a deactivation mechanism for RTKs, accumulated evidence is unveiling a positive signaling role for endosomally localized RTKs (Wang et al., 2002a) . However, nothing is known about the endosomal signalling of PDGFR. It is not known whether PDGFR can signal from endosomes and whether the endosomal signalling of PDGFR, if it exists, can produce any physiological outcomes. Based on the similarity between PDGFR and EGFR in their trafficking and signalling, it is possible that, like EGFR, PDGFR also signals from endosomes. To test this possibility, I applied the same approaches from our EGFR study to investigate the endosomal signaling of PDGFR (Wang et al., 2002a; Wang et al., 2002b; Pennock and Wang, 2003).

4.2 Selective activation of PDGFRs following their endocytosis into endosomes

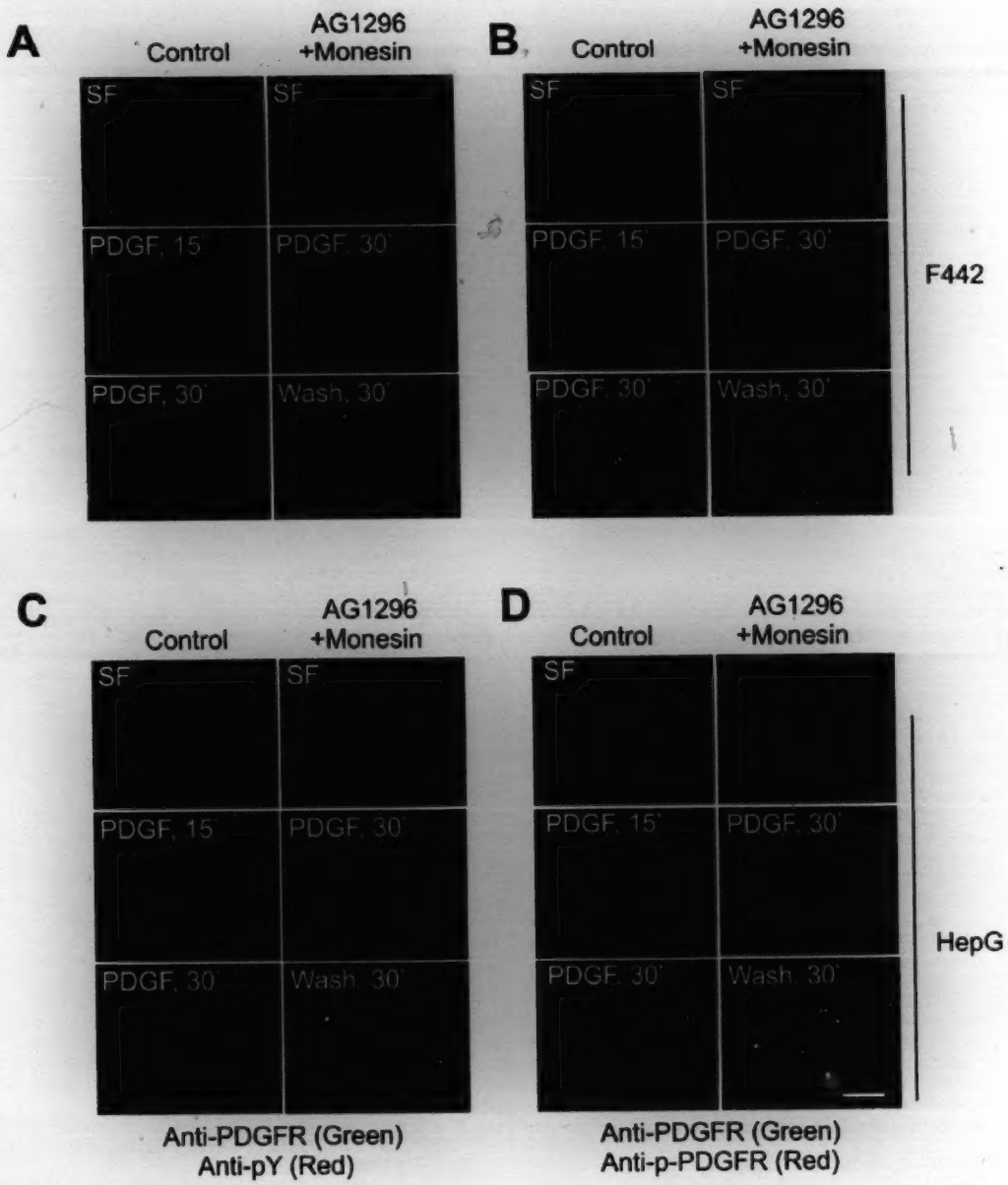
In Chapter 3, I established a system to selectively activate endosomal EGFR without the activation of the plasma membrane-associated EGFR. In this chapter, I apply the same approach to PDGFR. F442 cells and HepG cells that stably express human PDGFR were treated with AG1296 for 15 min and then stimulated with PDGF in the presence of monensin. After 30 min incubation with PDGF and monensin, the majority of PDGFR was internalized into endosomes (Figure 4.1). There is no overlap between PDGFR and pTyr immunostaining, indicating the endosome-associated PDGFRs were not phosphorylated upon internalization (Figure 4.1). For control cells treated only with

PDGF, PDGFRs were phosphorylated and internalized into endosomes as expected. To more specifically determine the phosphorylation status of endosome-associated PDGFR, F442 and HepG cells were double stained with anti-PDGFR and anti-phospho-PDGFR (p-PDGFR) antibodies following the treatment described above. The results confirmed that treatment of F442 and HepG cells with AG1296, PDGF and monensin induced internalization of non-phosphorylated PDGFR into endosomes (Figure 4.1).

To determine whether endosome-associated non-phosphorylated PDGFR could be activated, F442 and HepG cells were washed with medium. The endosome-associated PDGFR was significantly activated 30 min following wash (Figure 4.1). I did not observe any phosphorylation of plasma membrane-associated endosomes (Figure 4.1). These results suggest that I can specifically activate endosome-associated PDGFR.

These observations were further confirmed by subcellular fractionation experiments (Figure 4.2). It is well established that in the absence of ligand, PDGFR is localized on the plasma membrane where it is not phosphorylated. However, after induction by PDGF for 30 min, the vast majority of PDGFRs is phosphorylated and enriched in endosome fractions. PDGFR itself can therefore be used as a marker for both plasma membrane and endosomes. I showed that, for both F442 and HepG cells, in the absence of ligand PDGFR was primarily localized at the plasma membrane fraction and not phosphorylated; while PDGFR was primarily present in the endosome fraction and phosphorylated following standard PDGF stimulation (Figure 4.2). Additionally, early endosome autoantigen 1 (EEA1), a marker for endosomes, was highly enriched in our endosomes

Figure 4.1 Analysis by immunofluorescence of selective activation of PDGFR after its endocytosis into endosomes. (A) and (B), F442 cells were treated with AG1296 for 15 min and then stimulated with PDGF in the presence of monensin at 37 °C for 30 min. Some of the cells were then washed with PBS for three times and incubated with serum-free medium (SF) for 30 min (right panels), and F442 cells that were serum-starved or treated only with PDGF for 15 and 30 min at 37 °C were used as controls (left panels). PDGFR (green) and Tyr(P) (red)(A) or p-PDGFR (red)(B) localization was determined by indirect immunofluorescence. Co-localization of PDGFR/Tyr(P) (A) or PDGFR/p-PDGFR (B) is indicated by yellow. (C) and (D), HepG cells were treated with AG1296 for 15 min and then stimulated with PDGF in the presence of monensin at 37 °C for 30 min. Some of the cells were then washed with PBS for three times and incubated with serum-free medium for 30 min (right panels), and HepG cells that were serum-starved or treated only with PDGF for 15 and 30 min at 37 °C were used as controls (left panels). PDGFR (red) and Tyr(P) (green) (C) or p-PDGFR (green) (D) localization was determined by indirect immunofluorescence. Co-localization of PDGFR/Tyr(P) (C) or PDGFR/p-PDGFR (D) is indicated by yellow. Scale bar, 20 μm.


A
Control
AG1296
+Monesin
SF
SF
PDGF, 15'
PDGF, 30'
PDGF, 30'
Wash, 30'
B
Control
AG1296
+Monesin
SF
SF
PDGF, 15'
PDGF, 30'
PDGF, 30'
Wash, 30'
F442
C
Control
AG1296
+Monesin
SF
SF
PDGF, 15'
PDGF, 30'
PDGF, 30'
Wash, 30'
Anti-PDGFR (Green)
Anti-pY (Red)
D
Control
AG1296
+Monesin
SF
PDGF, 15'
PDGF, 30'
PDGF, 30'
Wash, 30'
Anti-PDGFR (Green)
Anti-p-PDGFR (Red)
HepG

fractions. Treatment with AG1296, monensin and PDGF resulted in the internalization of non-phosphorylated PDGFR into endosomes and the endosome-associated PDGFR was phosphorylated following washing and incubation in medium (Figure 4.2). Washing to remove AG1296 and monensin did not result in an increase of plasma membrane-associated PDGFR (Figure 4.2), which suggests there is no detectable recycling of PDGFR to the plasma membrane following the removal of monensin. More importantly, only very little phosphorylated PDGFR was detected at the plasma membrane (Figure 4.2). Together, these results suggest that I have established a system to specifically activate PDGFR in endosomes without meaningful PDGFR activation at the plasma membrane.

4.3 The level and duration of PDGFR activation in our established system

I next determined the level and duration of PDGFR activity in our system. HepG and F442 cells were treated with AG1296, PDGF and monensin as described above. Immunoblotting with anti-PDGFR and anti-p-PDGFR antibodies showed that before washing, no PDGFR was phosphorylated (Figure 4.3). Washing followed by incubation induced the phosphorylation of PDGFR. After washing for 30 min, phosphorylation of PDGFR reached a maximum that is 50% of the level following standard PDGF stimulation for 30 min. After washing for 2 h, the level of phosphorylated PDGFR was undetectable (Figure 4.3). This pattern of receptor dephosphorylation was similar to that following standard PDGF stimulation.

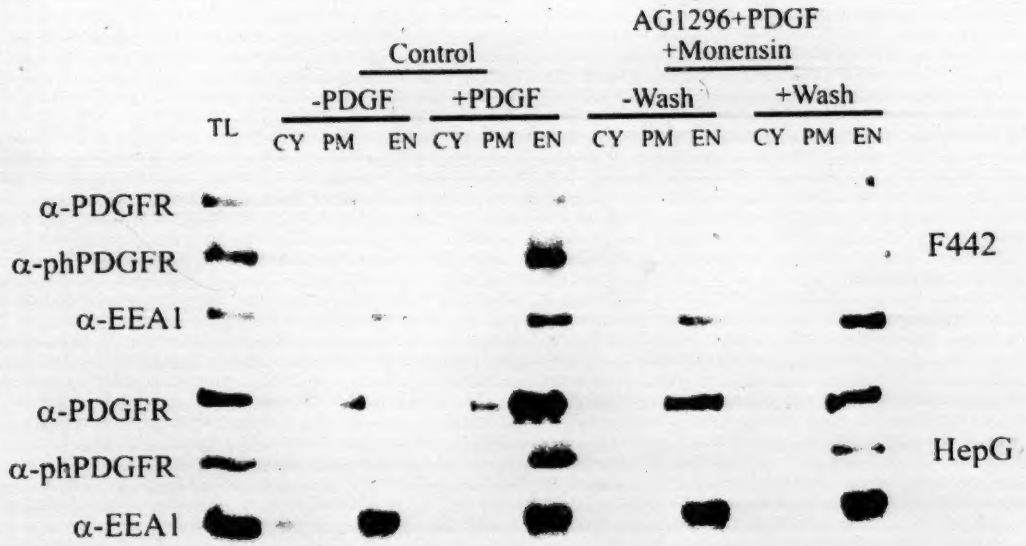


Figure 4.2 Further analysis of selective activation of endosome-associated PDGFR by subcellular fractionation. F442 and HepG cells were treated with AG1296, PDGF, and monensin as described above. The cells were then subcellular fractionated into the plasma membrane (PM), endosome (EN), and cytosol (CY) fractions. The subcellular fractions were subjected to immunoblotting with anti-PDGFR, anti-p-PDGFR, and anti-EEA1 antibodies. TL, total lysate.

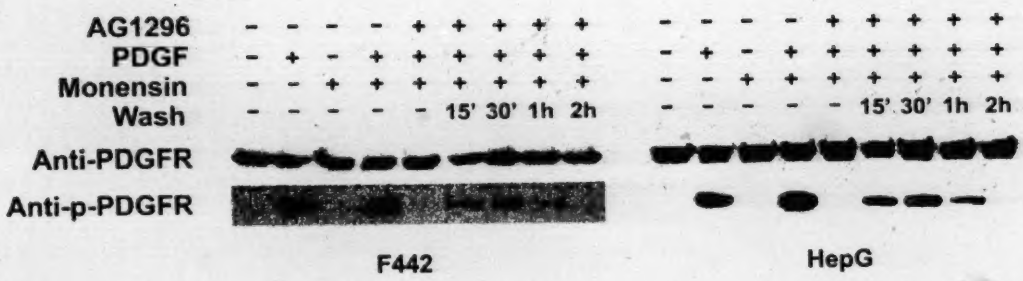


Figure 4.3 Immunoblot analysis of the level and duration of PDGFR phosphorylation following its selective activation in endosomes. HepG and F442 cells were treated with AG1296 and PDGF with or without monensin as described above. The cells were washed and then incubated with serum-free medium for indicated time. The cell lysates were subjected to immunoblot analysis with rabbit anti-PDGFR and anti-p-PDGFR antibodies.

4.4 Recruitment of signaling proteins into endosomes by activation of endosome-associated PDGFR

I next used this system to specifically study PDGFR-mediated signal transduction from endosomes. First I examined whether activation of endosome-associated PDGFR is able to interact with various signaling proteins to form a signaling complex in endosomes. HepG and F442 cells were treated with AG1296, followed by PDGF and monensin as described above. Cells lysates were immunoprecipitated with anti-PDGFR antibody and the immunoprecipitates were immunoblotted with antibodies to various signaling proteins. As shown in Figure 4.4, specific activation of endosome-associated PDGFR resulted in the association of PDGFR with Grb2, PLC-γ1 and the p85α subunit of PI3K. I can not clearly show the SHC bands in the immunoblot due to the similar molecular weight between IgG and SHC. To overcome this problem, I immunoprecipitated the total lysates with anti-SHC antibody. Immunoblotting the immunoprecipitates with anti-PDGFR antibody showed the association between PDGFR and SHC following the specific activation of endosome-associated PDGFR (Figure 4.4). Interestingly, while F442 cells archieved the maximal association between PDGFR and the signaling proteins within 15 to 30 min after washing, HepG cells archieved the maximal association between PDGFR and the signaling proteins with different kinetics. The above results indicate that direct activation of endosome-associated PDGFR is sufficient to recruit the major signaling proteins to endosomes to form the signaling complexes with PDGFR.

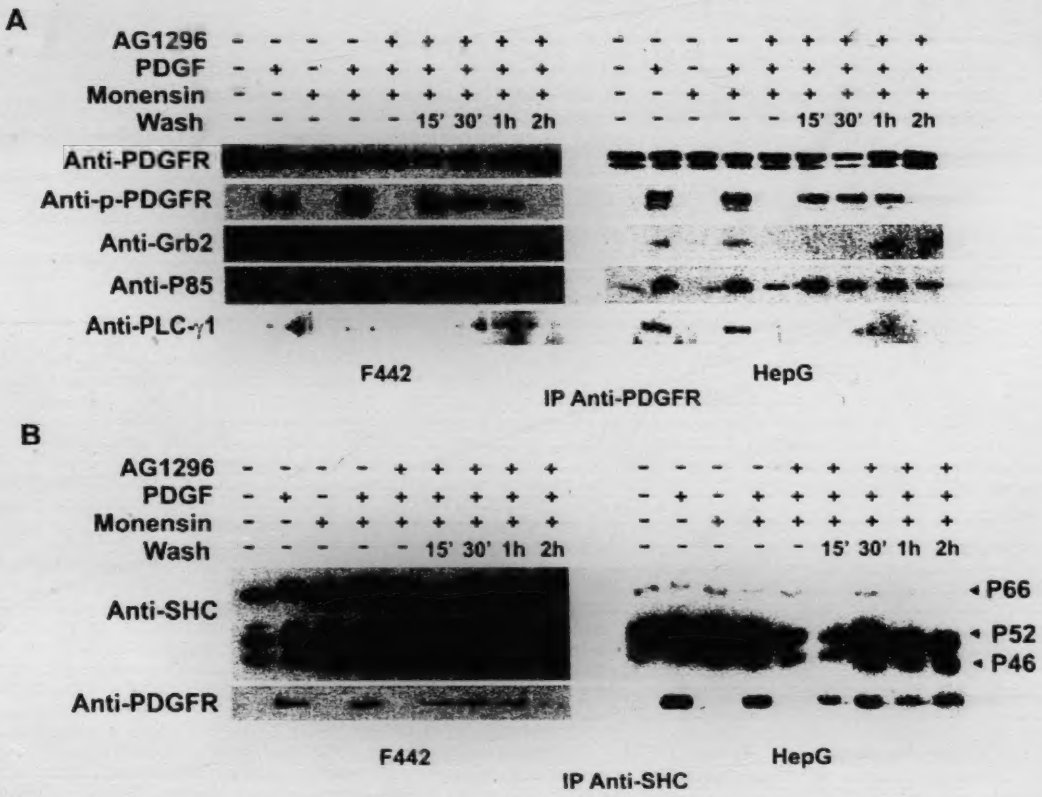


Figure 4.4 Interactions between signaling proteins and endosome-associated PDGFR in HepG and F442 cells. HepG and F442 cells were treated with AG1296 for 15 min and then stimulated with PDGF in the presence of monensin for 30 min. The cells were then washed with serum-free medium for indicated time. The cell lysates were immunoprecipitation (IP) with rabbit anti-PDGFR antibody (A) or rabbit anti-SHC antibody (B), followed by agarose-conjugated protein A. Immunoprecipitates were subjected to immunoblot analysis with rabbit anti-PDGFR, anti-p-PDGFR, anti-Grb2, anti-SHC, anti-p85α, and anti-PLC-γ1 antibodies. HepG and F442 cells that had been serum-starved or treated with PDGF, monensin, or PDGF plus monensin were used as controls.

4.5 Stimulation of various signaling pathways by endosomal signaling of PDGFR

I showed above that the specific activation of endosome-associated PDGFR is able to recruit major signaling proteins to endosomes. I further determined whether endosomal signaling of PDGFR is sufficient to stimulate various signaling pathways. It is known that following standard stimulation with PDGF, multiple signal transduction pathways are activated. These pathways include the Ras-ERK pathway, the PI3K-Akt pathway, and PLC-γ1 pathway. I showed that activation of endosome-associated PDGFR also leads to the phosphorylation of SHC (Figure 4.5A), ERK1/2 (Figure 4.5C), Akt (Figure 4.5D), PLC-γ1 (Figure 4.5E), as well as the activation of Ras as determined by association with the Ras-binding domain (RBD) of Raf (Figure 4.5B). The maximum phosphorylation for the activation level of endosome-associated PDGFR is approximately 50% of that following standard PDGF stimulation (Figure 4.3), and the activation levels of ERK1/2, Akt and PLC-γ1 was also approximately 50% of that following standard PDGF stimulation (Figure 4.5). These suggest that activation of endosome-associated PDGFR and standard activation of PDGFR are similarly effective on the induction of these signaling pathways.

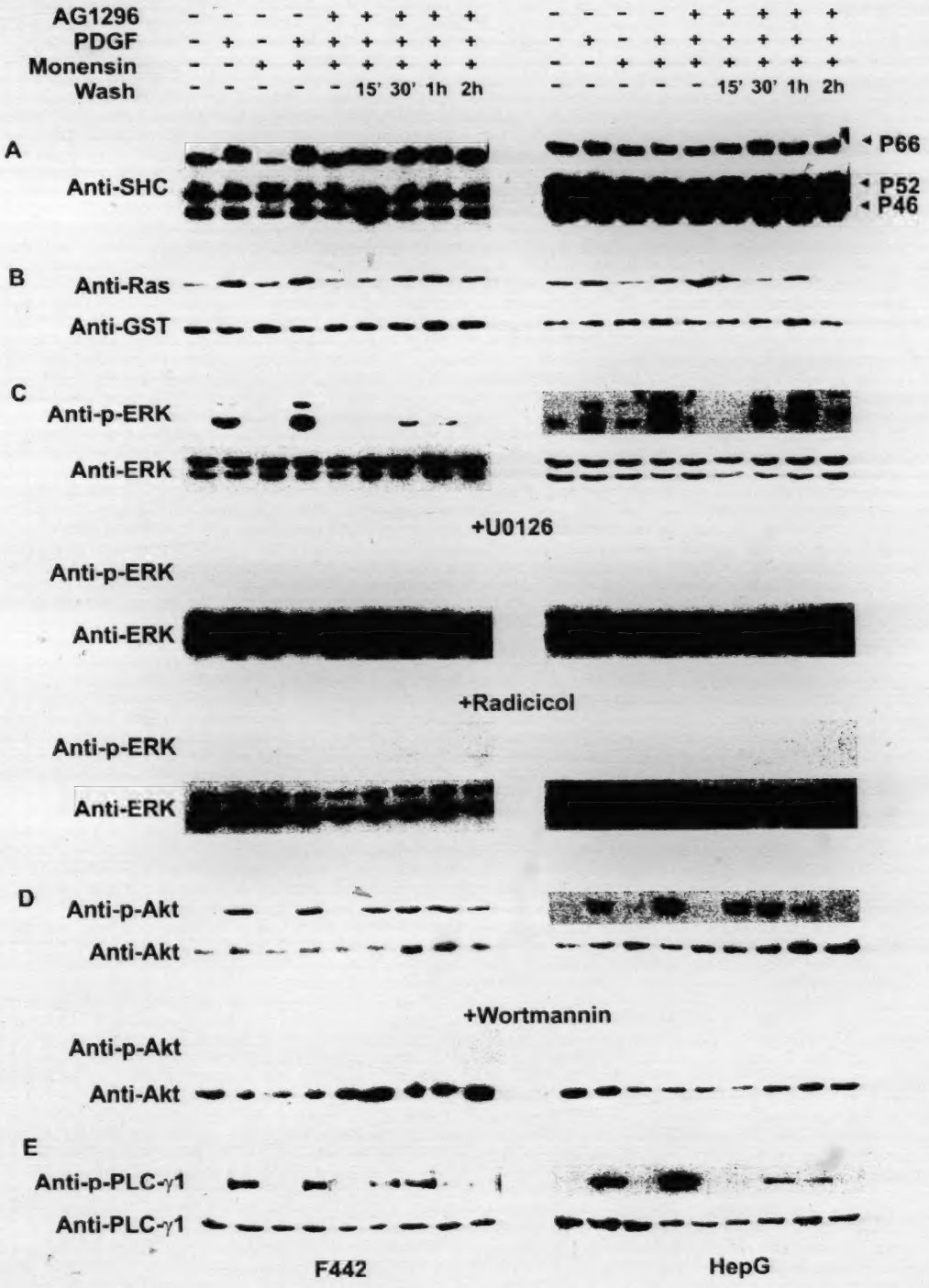
To determine whether the mechanism of downstream activation is the same as for standard PDGFR activation, I next examined the effect of specific inhibitors of ERK and Akt activation following stimulation of endosomal PDGFR. Our results showed that the MEK inhibitor U0126 blocked ERK phosphorylation following stimulation of endosomal PDGFR to the same extent as when stimulated by PDGFR initially at the cell surface

(Figure 4.5C). Moreover, depleting Raf with Radicicol also inhibited the activation of ERK stimulated by endosome-associated PDGFR to the same extent as that stimulated by the standard activated PDGFR (Figure 4.5C). Akt phosphorylation, induced following stimulation of endosomal PDGFR, is blocked by the PI3K inhibitor wortmannin, indicating that Akt activation is mediated by PI3K (Figure 4.5D). Together, these results indicate that endosomal signaling of PDGFR is sufficient to stimulate several signal transduction pathways implicated in cell mitogenesis and survival.

4.6 Support of cell survival by endosomal PDGFR signaling

Having positively established a signaling role for endosomally-activated PDGFR, I then determined whether these signals were of sufficient potency to produce a biological outcome. I first examined the effects of endosomal PDGFR signaling on serum-withdrawal induced apoptosis by using the TUNEL assay (Figure 4.6). HepG and F442 cells cultured in 10% FBS have a 5% basal rate of apoptosis. If the cells are then starved of serum for 48 h, this rate increases to 50%. As seen in Figure 4.6, a brief 1 h pulse of PDGF, administered after 36 h of starvation, provides a sufficient signal to save approximately half of them from apoptotic death. To determine whether an anti-apoptotic effect could be similarly elicited by activation of endosome-associated PDGFR, following 24 hours of starvation, HepG and F442 cells were stimulated with PDGF (10 ng/ml) for 30 min in the presence of AG1296, with or without monensin, to allow for the internalization of inactive PDGFR into endosomes. After the subsequent activation of endosome-associated PDGFR by washing and incubating with serum-free

Figure 4.5 Stimulation of various signal transduction pathways by activation of endosome-associated PDGFR. HepG and F442 cells were treated with AG1296 and PDGF with or without monensin as described above. The cells were washed and then incubated with serum-free medium for the indicated times. (A), activation of SHC. HepG and F442 cell lysates were subjected to immunoblot analysis with rabbit anti-SHC antibody. The activation of SHC is shown as the band shift. (B), activation of Ras. HepG and F442 cell lysates were incubated with GST-RBD conjugated with glutathione beads. The glutathione beads were then subjected to immunoblot analysis with mouse anti-Ras antibody. GST-RBD was stained by mouse anti-GST antibody to show equal loading. (C), activation of ERK. HepG and F442 cell lysates were subjected to immunoblot analysis with rabbit anti-phospho-ERK1/2 (p-ERK1/2) antibody. Total ERK1/2 was used as control. To determine the effects of Radicicol on the activation of ERK, HepG and F442 cells were treated with Radicicol (10 μM) for 40 h before the treatment described above. To determine the effects of U0126 on the activation of ERK, HepG and F442 cells were treated with U0126 (10 μM) for 30 min before the treatment described above. The cell lysates were subjected to immunoblot analysis with anti-p-ERK antibody. Anti-ERK blot was worked as loading control. (D), activation of Akt and its inhibition by wortmannin. Activation of Akt was determined by anti-phospho-Akt (p-Akt) antibody. To determine the effects of wortmannin on the activation of Akt, HepG and F442 cells were treated with wortmannin (100 nM) for 15 min before the treatment described above. The cell lysates were subjected to immunoblot analysis with anti-p-Akt antibody. Anti-Akt blot was used as loading control. (E), activation of PLC-γ1. Activation of PLC-γ1 was determined by immunoblotting of HepG and F442 cell lysates with anti-phospho-PLC-γ1 (p-PLC-γ1) antibody. Total PLC-γ1 was used as loading control.


AG1296
PDGF
Monensin
Wash
15' 30' 1h 2h
A
Anti-SHC
P66
P52
P46
B
Anti-Ras
Anti-GST
C
Anti-p-ERK
Anti-ERK
+U0126
Anti-p-ERK
Anti-ERK
+Radicicol
Anti-p-ERK
Anti-ERK
D
Anti-p-Akt
Anti-Akt
+Wortmannin
Anti-p-Akt
Anti-Akt
E
Anti-p-PLC-γ1
Anti-PLC-γ1
F442
HepG

medium for the remaining 12 h, the percentage of apoptotic cells was reduced to levels similar to that following the standard PDGF treatment, in this case a pulse of PDGF for 1 h (Figure 4.6). It is important to note that, in both cases, the pulse of PDGFR activation is limited to those receptors stimulated over the treatment period, and since the growth factor is thoroughly washed out following treatment, no more receptors will contribute to the anti-apoptotic "signal". This shows that endosomal PDGFR can transduce survival signals of physiological significance.

If the cells were treated with wortmannin, standard PDGF stimulation of 1 h could not promote the cell survival (Figure 4.6). This is expected, since wortmannin inhibits PI3K and thus prevents downstream signal transduction to inhibit apoptotic factors. To investigate whether the survival signals elicited from endosome-associated PDGFR originate from the same PI3K-Akt pathway, cells were pretreated with wortmannin (100 nM) and then treated 30 min with AG1296 and PDGF with or without monensin before washout. As can be seen in Figure 4.6, activation of endosome-associated PDGFR is unable to suppress apoptosis induced by serum-withdrawal. This indicates that similar to standard activation of PDGFR, activation of endosome-associated PDGFR supports cell survival by stimulating the PI3K-Akt pathway.

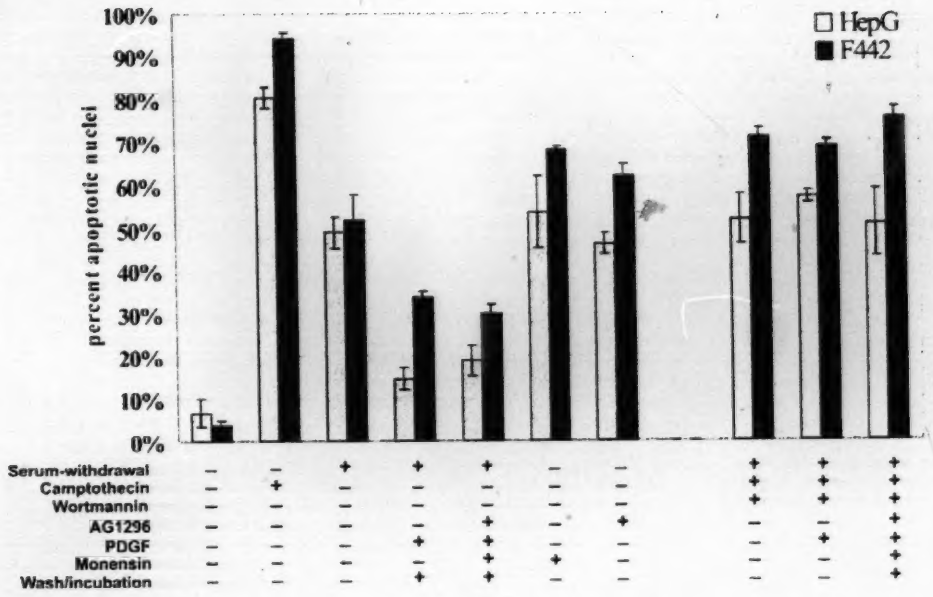


Figure 4.6 Activation of endosome-associated PDGFR and the effects on cell survival. HepG and F442 cells were either left in culture medium containing 10% fetal bovine serum with or without camptothecin or incubated in serum-free medium for 36 h to induce apoptosis. Some of the cells were pulsed with PDGF (10 ng/ml) for 1 h, followed by ligand washout. As additional controls, the cells were administered either monensin or AG1296 in place of PDGF. Some of the cells were treated with wortmannin (100 nM) to investigate the role of EGFR-activated PI3K in cell apoptosis. The percentage of apoptosis was determined by TUNEL assay; the results represent the accumulated data of triplicate experiments or 1500 counted cells

4.7 The effect of endosome-associated PDGFR on cell proliferation

Finally, I determined whether endosomal PDGFR signaling is sufficient to stimulate cell proliferation. It was shown recently that the sustained requirement (more than 8 h) for PDGF to induce cell proliferation can be replaced by two shorter pulses of PDGF (Jones and Kazlauskas, 2001). In quiescent cells, the two pulses must coincide first with re-entry of the cells into the G1 cell cycle, and then again during late G1 – a time close to the restriction point. In many cell types including our own, the times at which mitogen are required are spaced approximately 7-9 h apart. These findings allowed us to test whether two pulses of endosomal PDGFR signaling is sufficient to stimulate cell proliferation. Indeed our laboratory has shown recently that two pulses of endosomal EGFR signaling are sufficient to stimulate cell proliferation (Pennock and Wang, 2003).

Cell proliferation (DNA synthesis) was assayed by BrdU incorporation into cells. As shown in Figure 4.7, one pulse of endosomal PDGFR signaling is insufficient to stimulate S phase entry. However, two pulses of endosomal PDGFR signaling can drive cells into S-phase with equal efficacy as two-pulses of standard activation from PDGFR, in both HepG and F442 cells. Moreover, the percent of proliferating cells following two-pulse treatment, whether from standard activated or endosomally activated PDGFR, is similar to cells treated continually over 8 h with either PDGF or 10% FBS. This demonstrates that PDGFR signals, derived exclusively from the endosome, can lead to cell proliferation; furthermore, the potency of the endosomal signal is kinetically similar to that derived from the standard PDGFR activation.

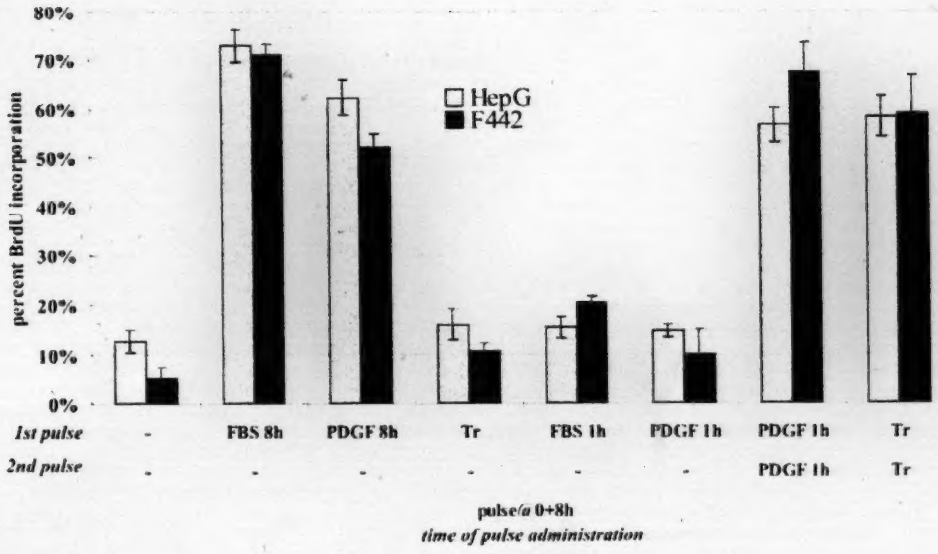


Figure 4.7 Activation of endosome-associated PDGFR and the effects on cell proliferation. HepG and F442 cells were serum-starved for 24 h. The cells were then stimulated with PDGF (standard PDGF stimulation) or treated with one or two pulses of AG1296 plus PDGF and monensin. After pulsing, the cells were incubated with serum-free medium in the presence of BrdUrd. BrdUrd incorporation was assayed as described under "Materials and Methods." Tr, cells were treated with AG1296 and PDGF (10 ng/ml) with monensin followed by washing and incubation as described under "Materials and Methods."

4.8 Summary

In summary, our results indicate that, to a large extent, PDGFR-mediated signaling from endosomes is very similar to the standard PDGFR-mediated signaling that includes the signaling from both the plasma membrane and endosomes. Based on our observations, the time frame of PDGFR endocytosis at the plasma membrane lasts no longer than 15 min following PDGF binding. The remainder of PDGFR's signaling activity must therefore occur in the endosomes, and persist a much longer span of time.

The similar results obtained for both PDGFR and EGFR suggest that our recently established system to specifically generate endosomal signaling of EGFR may be suitable for examining many other RTKs (Wang *et al.*, 2002a; Pennock and Wang, 2003). More importantly, our results suggest that the concept of endosomal signaling may apply to all RTKs.

Chapter 5

Regulation of EGF-induced phospholipase C-γ1 translocation by its SH2 and PH domains

The majority of the information presented in this chapeter has been published in Wang, Y. and Z.Wang. 2003. Regulation of EGF-induced phospholipase C-γ1 translocation and activation by its SH2 and PH domains. *Traffic* 4: 618-630. It has been adapted into this Thesis with permission.

5.1 Overview

PLC-γ1 plays a significant role in mediating GF-induced cell signaling (Todderud et al., 1990). In response to GF stimulation, PLC-γ1 forms a complex with the activated GF receptor (GFR) (Kim et al., 1990; Margolis et al., 1990; Meisenhelder et al., 1989; Anderson et al., 1990). Complex formation leads to phosphorylation of PLC-γ1 on tyrosine residues and an increase in its enzymatic activity (Kim et al., 1991; Ronnstrand et al., 1992; Rotin et al., 1992). Active PLC-γ1 hydrolyzes PtdIns(4,5)P_2 to form the second messengers $InsP_3$ and DAG, which mobilizes intracellular calcium and activates PKC, respectively (Burgess et al., 1984; Berridge and Irvine, 1984; Nishizuka, 1992).

GF stimulates the sub translocation of PLC-γ1, which plays an important role in modulating the function of PLC-γ1. However, the translocation pattern of PLC-γ1 following GF stimulation is still not clear. Initially, it was suggested that PLC-γ1 translocates from the cytosol to the plasma membrane to form complex with GFR (Kim et al., 1990). Recently, it was shown that GFP-tagged PLC-γ1 translocates to the endosomes following its initial translocation from cytosol to the plasma membrane in response to EGF (Ye et al., 2002). Some signaling proteins including SHC, Grb2, and GAP have been shown to co-translocate with GFRs from the plasma membrane to endosomes and to mediate the GFR signaling from endosomes (Pawson, 1995; Falasca et al., 1998). As a consequence, it would be very interesting to see whether PLC-γ1 remains phosphorylated and functional in endosomes. Moreover, another recent report showed

that PLC-γ1 translocated to the nucleus following NGF stimulation (Pei et al., 1996). Thus, the GF-induced translocation pattern of PLC-γ1 remains to be fully established.

Another intriguing question is which domain of PLC-γ1 is responsible for regulating the translocation of PLC-γ1 in response to GF. PLC-γ1 contains two SH2 domains, one SH3 domain, one split PH domain and one complete PH domain. Each of these domains can fold into a compact and functional module independent of surrounding sequences (Itoh et al., 1996). All of these domains have been implicated in controlling localization and GF-induced translocation of PLC-γ1 (Itoh et al., 1996). Initially, it was shown that the translocation of PLC-γ1 from cytosol to the plasma membrane is regulated by the interactions between the SH2 domains of PLC-γ1 and the phosphotyrosine residues of GFRs (Cantley et al., 1991). Recently, it was reported that EGF-induced membrane targeting of PLC-γ1 is mediated by the PLC-γ1 PH domain (Bae et al., 1998). However, in contrast, two more recent publications showed that the translocation of PLC-γ1 to the plasma membrane required the functional SH2 domains and was not affected by mutations in the PH domain or inhibition of PI3K (Ye et al., 2002; Wang et al., 2001). These data are clearly at odds with the finding of Falasca *et al.* (Bae et al., 1998).

The issue regarding the regulation of GF-induced translocation of PLC-γ1 is further complicated by the following findings. It has been reported that the C-SH2 domain of PLC-γ1 binds to F-actin (Pei et al., 1996), tubulin (Itoh et al., 1996) and PtdIns(3,4,5)P_3 (Bae et al., 1998) *in vitro*, which suggests that C-SH2 domain may be

involved in the localization of PLC-γ1 to the cell cytoskeleton. PLC-γ1 SH3 domain has been shown to target PLC-γ1 to the cytoskeleton and may function to regulate actin polymerization (Bar-Sagi et al., 1993).

In light of these conflicting results, I studied the EGF-induced translocation of PLC-γ1 and the molecular mechanisms regulating the translocation.

5.2 EGF-induced translocation of endogenous PLC-γ1

While GFP-tagged PLC-γ1 is localized to the endosomes following EGF stimulation, it is not clear whether this is truly a physiological phenomenon or simply due to the overexpression of the protein. To answer this question, I determined the translocation pattern of endogenous PLC-γ1 in response to EGF. In BT-20 cells, PLC-γ1 was non-phosphorylated and primarily localized to the cytosol without EGF stimulation (Figure 5.1A, 1B). After EGF stimulation for 5 min, a portion of PLC-γ1 was phosphorylated and co-localized to the plasma membrane with EGFR (Figure 5.1C). Following stimulation for 30 min, phosphorylated PLC-γ1 was co-localized with EGFR to endosomes, suggesting that endogenous PLC-γ1 translocated to endosomes in phosphorylated form (Figure 5.1D). I did not observe any localization of PLC-γ1 in nucleus before and after EGF stimulation (Figure 5.1). To further determine the nuclear localization of PLC-γ1, I purified nuclei of COS-7 and BT20 cells. Immunoblotting with an anti-PLC-γ1 antibody showed that PLC-γ1 was not detected in nuclear fractions with or without EGF stimulation (Figure 5.1E). The purity of the nuclear fractions was

determined by anti-tubulin (cytoplasm marker) and anti-lamin (nuclei marker) antibodies (Figure 5.1E).

5.3 EGF-induced translocation of wtPLC-γ1

To more precisely map EGF-induced PLC-γ1 translocation, wild-type PLC-γ1 tagged with YFP at its N-terminus (wtPLC-γ1) was constructed (Figure 5.2A) and transiently expressed in COS-7 cells. Due to its intrinsic fluorescence, the dynamic subcellular localization of this wtPLC-γ1 could be observed in living cells. Analysis of the transfected cells showed that approximately 30% of the cells express YFP at different levels. Cells expressing an intermediate level of fluorescence were chosen for study. As COS-7 cells have little endogenous PLC-γ1, the expression level of YFP-PLC-γ1 is at least 3-fold more than the endogenous one. In the real-time fluorescence experiments (Figure 5.2B, Supplementary data: Video 1), I confirmed previous report that EGF stimulates the translocation of wtPLC-γ1 from cytosol to the plasma membrane and then to endosomes. wtPLC-γ1 was not detected in the nucleus before or after EGF stimulation. Indirect immunofluorescence further showed that wtPLC-γ1 co-localized with phosphorylated EGFR (p-EGFR) in endosomes following EGF stimulation (Figure 5.2C). Subcellular fractionation and immunoblotting showed that wtPLC-γ1 was mostly in the cytosol before EGF stimulation (Figure 5.2D). After 1-min EGF stimulation, approximately 15% of cytosolic wtPLC-γ1 was translocated to the membrane (Figure 5.2D and Figure 5.3D). Membrane localization of wtPLC-γ1 was quite pronounced following EGF stimulation

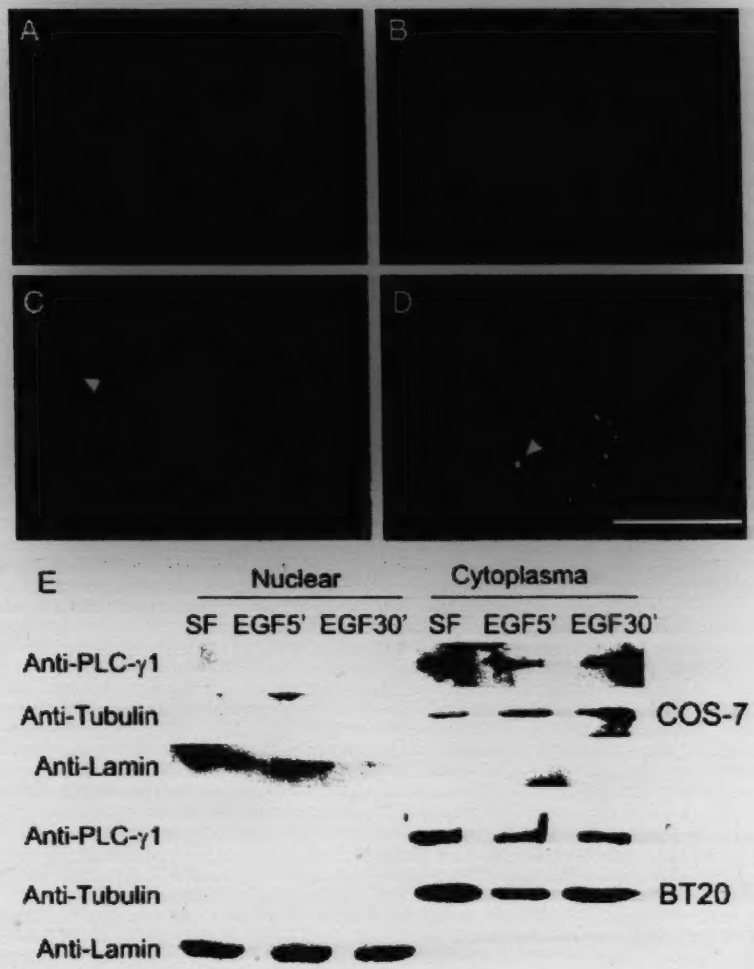


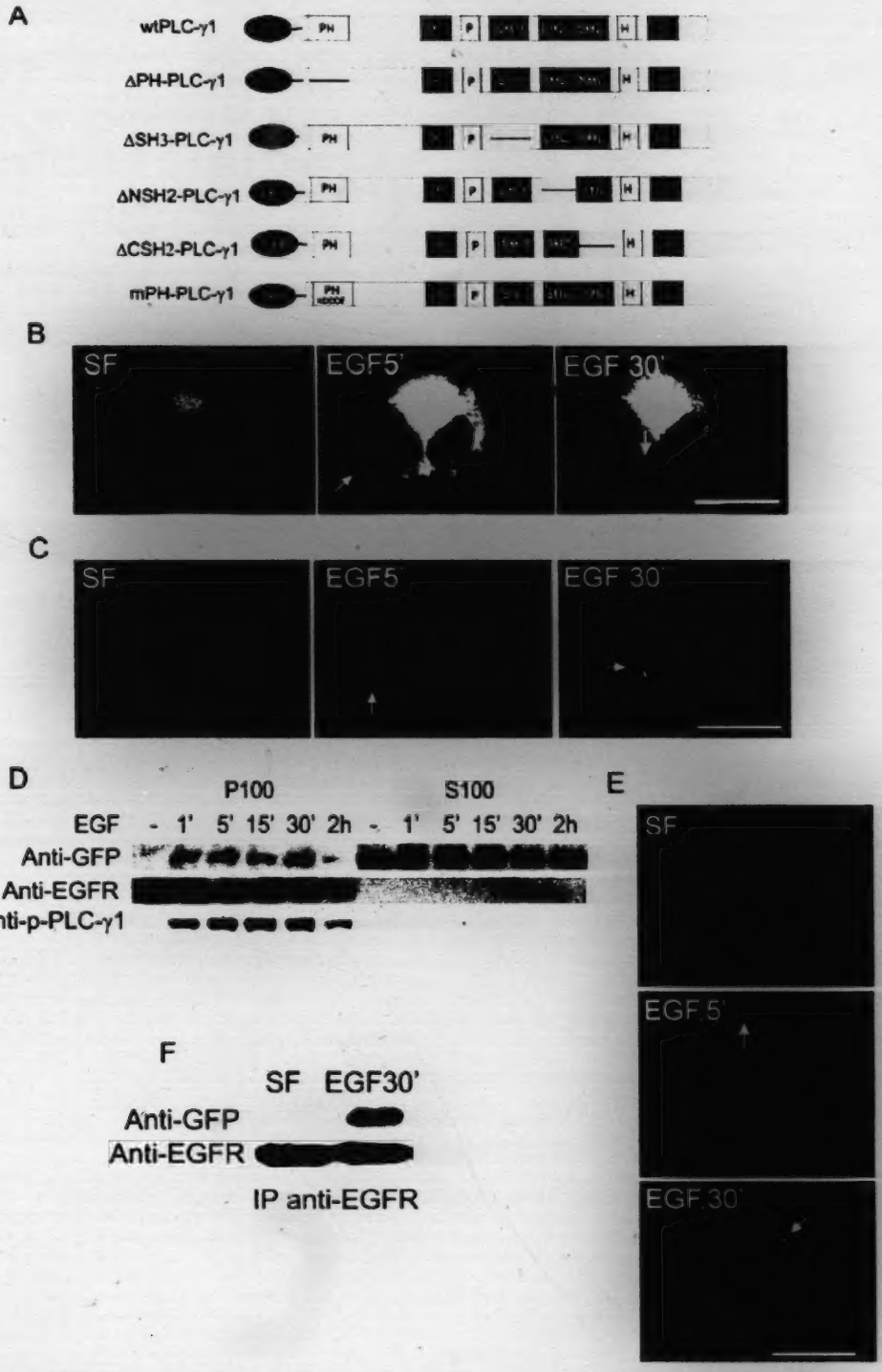
Figure 5.1 EGF-induced translocation and phosphorylation of endogenous PLC-γ1. (A–D) immunofluorescence analysis. BT-20 cells were serum-deprived (A andB), or incubated with 100 ng/ml EGF for 5 min (C) and 30 min (D). EGFR (red) and PLC-γ1 (A, green)/p-PLC-γ1 (B-D, green) localization was determined by indirect immunofluorescence. Co-localization (yellow) of EGFR and p-PLC-γ1 is indicated by arrowhead. Size bar = 20 μm (E) Sub-cellular fractionation. COS-7 and BT20 cells were stimulated with EGF and sub-cellular fractionated into nuclear and cytoplasma fractions. The localization of PLC-γ1 was determined by immunoblotting with anti-PLC-γ1 antibody. Immunoblotting of Tubulin and Lamin was used as controls.

for 5 min and remained stable at a high level for up to 30 min. After a prolonged stimulation of EGF for 2 h, membrane-associated wtPLC-γ1 decreased significantly. Moreover, immunoblotting with anti-phospho-PLC-γ1 (p-PLC-γ1) antibody showed that EGF stimulates strong phosphorylation of wtPLC-γ1 and the phosphorylated PLC-γ1 was primarily associated with the membrane (Figure 5.2D). Immunofluorescence analysis further revealed that EGF stimulation resulted in strong phosphorylation of PLC-γ1 that was initially associated with the plasma membrane and then associated with endosomes (Figure 5.2E). Co-immunoprecipitation experiments showed that EGF stimulated strong association between wtPLC-γ1 and EGFR (Figure 5.2F).

5.4 The role of the individual domains in the regulation of EGF-induced PLC-γ1 translocation

I next determined the role of individual PLC-γ1 domains in the regulation of EGF-induced translocation of PLC-γ1 by using a battery of domain deletion mutants of PLC-γ1 including ΔNSH2-PLC-γ1, ΔCSH2-PLC-γ1, ΔPH-PLC-γ1, and ΔSH3-PLC-γ1 (Figure 5.2A). COS-7 cells were transfected with these mutants and EGF-induced translocation of these mutants was monitored in living cells (Figure 5.3A, Supplementary data: Video 2-5) and by indirect immunofluorescence (Figure 5.3B). Following EGF stimulation for 5 min, the translocation of PLC-γ1 to the plasma membrane was impaired in the two SH2 deletion mutants, but unaffected in the SH3 or PH domain deletion mutants (Figure 5.3A & 5.3B). These results suggest that both SH2 domains of PLC-γ1 are solely responsible for the initial plasma membrane targeting of PLC-γ1. Following

Figure 5.2: EGF-induced translocation and activation of wtPLC-γ1. (A) Schematic representation of various PLC-γ1 constructs. The catalytic domain is composed of X and Y regions. (B–E) COS-7 cells were transfected with wtPLC-γ1 and used for the various analyses. (B) The real time images of wtPLC-γ1 before and after EGF stimulation. The membrane and endosome localization are indicated by arrows. (C) Indirect immunofluorescence. The colocalization (yellow) of phospho-EGFR (red) and intrinsic fluorescence of wtPLC-γ1 (green) is indicated by arrows. (D) The total cell homogenates were subcellularly fractionated into membrane (P100) and cytosol (S100) and immunoblotted with anti-GFP (indication of wt PLC-γ1), anti-EGFR and anti-p-PLC-γ1 antibodies. (E) Indirect immunofluorescence. The colocalization (yellow) of p-PLC-γ1 (red) and intrinsic fluorescence of wtPLC-γ1 (green) is indicated by arrow. Size bar = 20 μm. (F) Co-immunoprecipitation of EGFR and wtPLC-γ1 with monoclonal anti-EGFR antibody Ab-1, and the resulting immunoprecipitates were subjected to immunoblotting with a polyclonal anti-GFP antibody or polyclonal anti-EGFR antibody. Size bar = 20 μm.


A
wtPLC-γ1
ΔPH-PLC-γ1
ΔSH3-PLC-γ1
ΔNSH2-PLC-γ1
ΔCSH2-PLC-γ1
mPH-PLC-γ1
B
SF
EGF5'
EGF 30'
C
SF
EGF5'
EGF 30'
D
P100
S100
EGF - 1' 5' 15' 30' 2h - 1' 5' 15' 30' 2h
Anti-GFP
Anti-EGFR
Anti-p-PLC-γ1
E
SF
EGF 5'
EGF 30'
F
SF EGF30'
Anti-GFP
Anti-EGFR
IP anti-EGFR

EGF stimulation for 30 min, the plasma membrane localized ΔSH3-PLC-γ1 was translocated to endosomes. Interestingly, while the plasma membrane associated ΔNPH-PLC-γ1 disappeared after EGF stimulation for 30 min, only a small portion of ΔNPH-PLC-γ1 was localized to endosomes (Figure 5.3A-B), which indicates the disassociation of ΔNPH-PLC-γ1 from the membrane (the plasma membrane or endosome membrane), rather than the inhibition of the translocation of ΔNPH-PLC-γ1 from the plasma membrane to endosomes. Both the ΔNSH2-PLC-γ1 and ΔCSH2-PLC-γ1 remain cytosolic (Figure 5.3A-B). These fluorescence results were confirmed by subcellular fractionation and immunoblotting (Figure 5.3C-D and Table 1). At 5 min, the membrane-bound ΔNPH-PLC-γ1 was at a level similar to that of wtPLC-γ1 and ΔSH3-PLC-γ1, however, at 15 and 30 min the membrane-bound ΔNPH-PLC-γ1 was significantly lower than that of wtPLC-γ1 and ΔSH3-PLC-γ1. The differences and similarities were confirmed by statistical analysis (Table 5.1). Together, these results suggest that the PH domain of PLC-γ1 may function in assisting the stabilization of PLC-γ1 at the membrane.

5.5 The role of the PH domain in regulating PLC-γ1 activity

I next determined the role of various PLC-γ1 domains in regulating the activity of PLC-γ1. COS7 cells were transfected with various domain deletion mutants of PLC-γ1 including ΔNSH2-PLC-γ1, ΔCSH2-PLC-γ1, ΔPH-PLC-γ1, and ΔSH3-PLC-γ1. Both fluorescence and immunoblotting results indicated that the deletion of PLC-γ1 SH3 domain did not affect EGF-induced phosphorylation of PLC-γ1 (Figure 5.4A-B). Neither

Figure 5.3 EGF-induced translocation of various PLC-γ1 deletion mutants. COS-7 cells were transiently transfected with ΔNSH2-PLC-γ1, ΔCSH2-PLC-γ1, ΔPH-PLC-γ1, and ΔSH3-PLC-γ1, and serum starved for 24 h. (A) The real time images of various PLC-γ1 deletion mutants before and after EGF stimulation. The membrane and endosome localization are indicated by arrow. (B) Indirect immunofluorescence. The colocalization (yellow) of phospho-EGFR (Red) and intrinsic fluorescence of various PLC-γ1 deletion mutants (green) is indicated by arrow. Size bar = 20 μm. (C) The total cell homogenates were subcellularly fractionated into membrane (P100) and cytosol (S100) and immunoblotted with anti-GFP, anti-EGFR and anti-p-PLC-γ1 antibodies. (D) Quantification of the results from C and Figure 5.2D.

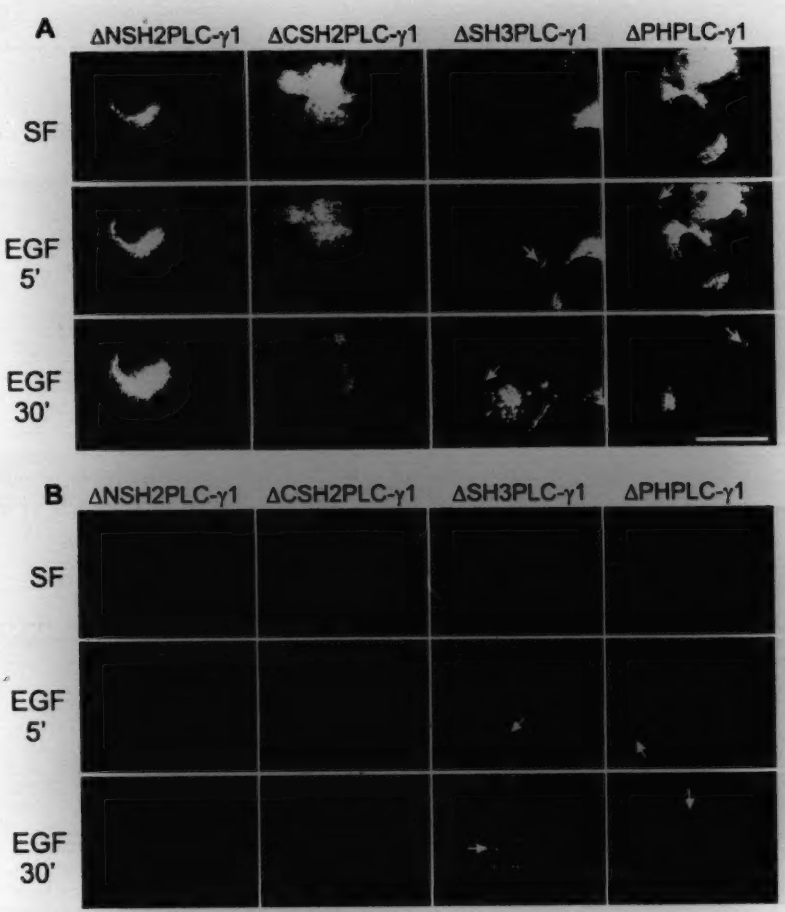

A
ΔNSH2PLC-γ1
ΔCSH2PLC-γ1
ΔSH3PLC-γ1
ΔPHPLC-γ1
SF
EGF 5'
EGF 30'
B
ΔNSH2PLC-γ1
ΔCSH2PLC-γ1
ΔSH3PLC-γ1
ΔPHPLC-γ1
SF
EGF 5'
EGF 30'
C
P100
S100
EGF - 1' 5' 15' 30' 2h - 1' 5' 5' 30' 2h
Anti-GFP
Anti-EGFR
ΔNSH2PLC-γ1
Anti-GFP
Anti-EGFR
ΔCSH2PLC-γ1
Anti-GFP
Anti-EGFR
ΔSH3PLC-γ1
Anti-GFP
Anti-EGFR
ΔPHPLC-γ1
D
Membrane association
P100/(P100+S100)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
5F
EGF 1'
EGF 5'
EGF 15'
EGF 30'
EGF 120'
WtPLC-γ1
ΔPH-PLC-γ1
ΔSH3-PLC-γ1
ΔNSH2-PLC-γ1
ΔCSH2-PLC-γ1

Table 5.1 Statistical analysis of the results from Figure 5.3D. Each group has 3 samples, and the difference between 2 groups was determined by Student's t-test

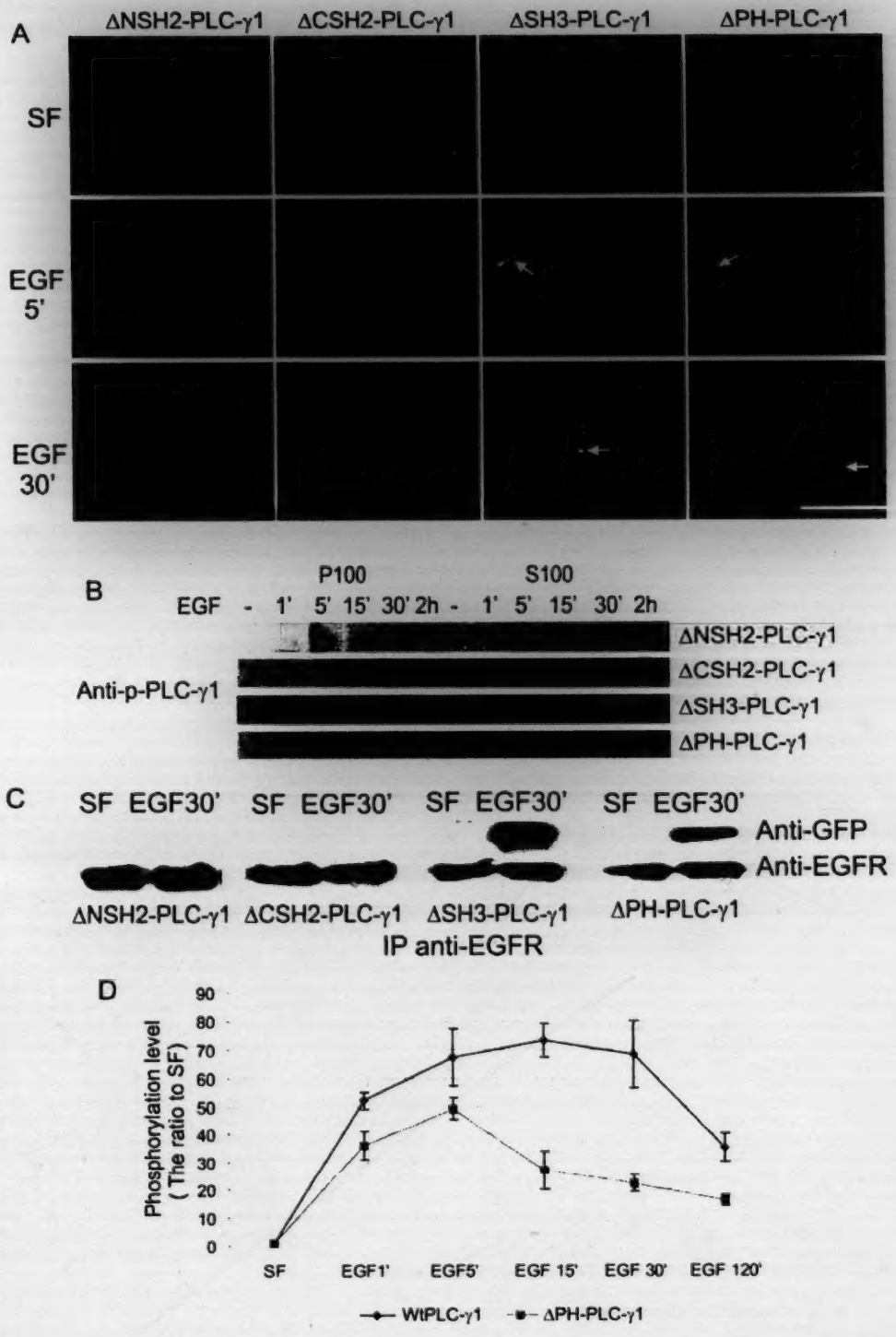
	wtPLC-γ1	ΔPH-PLC-γ1	ΔSH3-PLC-γ1	ΔNSH2-PLC-γ1	ΔCSH2-PLC-γ1
EGF 5 min					
wtPLC-γ1		p > 0.05	p > 0.05	**p < 0.01**	**p < 0.01**
ΔPH-PLC-γ1	p > 0.05		p > 0.05	**p < 0.01**	*p < 0.05*
ΔSH3-PLC-γ1	p > 0.05	p > 0.05		**p < 0.01**	**p < 0.01**
ΔNSH2-PLC-γ1	**p < 0.01**	**p < 0.01**	**p < 0.01**		p > 0.05
ΔCSH2-PLC-γ1	**p < 0.01**	*p < 0.05*	**p < 0.01**	p > 0.05	
EGF 15 min					
wtPLC-γ1		*p < 0.05*	p > 0.05	**p < 0.01**	**p < 0.01**
ΔPH-PLC-γ1	*p < 0.05*		*p < 0.05*	**p < 0.01**	**p < 0.01**
ΔSH3-PLC-γ1	p > 0.05	*p < 0.05*		**p < 0.01**	**p < 0.01**
ΔNSH2-PLC-γ1	**p < 0.01**	**p < 0.01**	**p < 0.01**		p > 0.05
ΔCSH2-PLC-γ1	**p < 0.01**	**p < 0.01**	**p < 0.01**	p > 0.05	
EGF 30 min					
wtPLC-γ1		**p < 0.01**	p > 0.05	**p < 0.01**	**p < 0.01**
ΔPH-PLC-γ1	**p < 0.01**		*p < 0.05*	**p < 0.01**	**p < 0.01**
ΔSH3-PLC-γ1	p > 0.05	*p < 0.05*		**p < 0.01**	**p < 0.01**
ΔNSH2-PLC-γ1	**p < 0.01**	**p < 0.01**	**p < 0.01**		p > 0.05
ΔCSH2-PLC-γ1	**p < 0.01**	**p < 0.01**	**p < 0.01**	p > 0.05	

did the deletion of the SH3 domain block EGF-induced association of PLC-γ1 with EGFR following EGF stimulation (Figure 5.4C). However, the two SH2 domains are required for EGF-induced activation of PLC-γ1 and its association with EGFR (Figure 5.4A-C). Interestingly, while the deletion of PH domain did not affect the initial phosphorylation of PLC-γ1 and the interaction between PLC-γ1 and EGFR, it did result in the quick decrease of PLC-γ1 phosphorylation following EGF stimulation for 15-30 min (Figure 5.4A-D). These results indicate that the PH domain is required for sustained activation of PLC-γ1.

5.6 The role of the PH domain in PLC-γ1 membrane association and activation is dependent on PI3K activity

It was shown that in response to EGF the PH domain targets PLC-γ1 to the plasma membrane in a PI3K-dependent pathway (Pei et al., 1996). While I showed above that the PH domain is not required for the initial translocation of PLC-γ1 to the plasma membrane in response to EGF, it is possible that the PH domain plays a role in stabilizing PLC-γ1 in the membrane and that the maintenance of high PLC-γ1 activity could be dependent on PI3K activity. To examine this possibility, COS-7 cells were transfected with wtPLC-γ1 and treated with wortmannin. Indirect immunofluorescence and subcellular fractionation showed that treatment with wortmannin did not affect EGF-induced initial translocation of wtPLC-γ1 from cytosol to the plasma membrane, however, increased the dissociation of wtPLC-γ1 from the membrane in the later time

Figure 5.4 The effects of PH domain on EGF-induced activation of PLC-γ1. COS-7 cells were transiently transfected with ΔNSH2-PLC-γ1, ΔCSH2-PLC-γ1, ΔPH-PLC-γ1, and ΔSH3-PLC-γ1. (A) Indirect immunofluorescence of EGF-induced phosphorylation of PLC-γ1. The colocalization (yellow) of p-PLC-γ1 (red) and intrinsic fluorescence of various PLC-γ1 deletion mutants (green) is indicated by the arrow. (B) The cells were subcellularly fractionated and immuno-blotted with anti-p-PLC-γ1 antibodies. (C) Co-immunoprecipitation of EGFR and PLC-γ1 with monoclonal anti-EGFR antibody Ab-1. The immunoprecipitates were immuno-blotted with a polyclonal anti-GFP antibody or anti-EGFR antibody. (D) Quantitation of the data from B and Figure 5.2D.


A
ΔNSH2-PLC-γ1
ΔCSH2-PLC-γ1
ΔSH3-PLC-γ1
ΔPH-PLC-γ1
SF
EGF 5'
EGF 30'
B
P100
S100
EGF - 1' 5' 15' 30' 2h - 1' 5' 15' 30' 2h
Anti-p-PLC-γ1
ΔNSH2-PLC-γ1
ΔCSH2-PLC-γ1
ΔSH3-PLC-γ1
ΔPH-PLC-γ1
C
SF EGF30' SF EGF30' SF EGF30' SF EGF30'
Anti-GFP
Anti-EGFR
ΔNSH2-PLC-γ1 ΔCSH2-PLC-γ1 ΔSH3-PLC-γ1 ΔPH-PLC-γ1
IP anti-EGFR
D
Phosphorylation level (The ratio to SF)
90
80
70
60
50
40
30
20
10
0
SF
EGF1'
EGF5'
EGF 15'
EGF 30'
EGF 120'
WtPLC-γ1
ΔPH-PLC-γ1

point (Figure 5.5A-C). In fact, EGF-induced translocation pattern of wtPLC-γ1 in the presence of wortmannin is identical to that of ΔNPH-PLC-γ1 in the absence of wortmannin (Figure 5.5C). The efficacy of wortmannin in inhibiting PI3K was confirmed by Akt phosphorylation (Figure 5.5B). While in the absence of wortmannin, EGF stimulates strong Akt phosphorylation (Figure 5.5B control). Treatment with wortmannin (100 nM) completely inhibited EGF-induced Akt phosphorylation (Figure 5.5B wortmannin). Moreover, treatment with wortmannin did not alter the translocation pattern of ΔNPH-PLC-γ1 (Figure 5.5D). These results suggest that the role of PH domain in stabilizing PLC-γ1 at the membrane and in maintaining PLC-γ1 activity is dependent on PI3K activity.

5.7 EGF-stimulated translocation of the mutant PLC-γ1 with point mutation at its PH domain

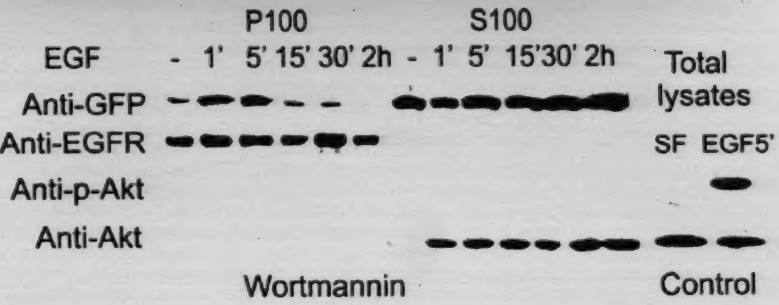
To demonstrate that the effects of PH domain deletion on EGF-stimulated translocation of PLC-γ1 is due to the lack of PH domain rather than the conformational change induced by the deletion, I generated a mutant PLC-γ1 with point mutation at its N terminal PH domain (mPH-PLC-γ1). It was shown that mutation of 64RGADK68 to 64KDDDE68 disrupted the function of the PH domain (Matsuda et al., 2001; Falasca et al., 1998). I showed that in COS-7 cells transfected with mPH-PLC-γ1, EGF induced translocation of mPH-PLC-γ1 shared the same pattern as ΔNPH-PLC-γ1 (Figure 5.6). Following EGF stimulation for 5 min, mPH-PLC-γ1 translocated from the cytosol to the

Figure 5.5 The effects of wortmannin on the translocation of PLC-γ1. (A–C) COS-7 cells were transiently transfected with wtPLC-γ1 and serum starved for 24 h. The cells were treated with wortmannin (100 nM) for 10 min and then stimulated with EGF for 5 or 30 min. (A) Indirect immuno-fluorescence. The colocalization (yellow) of EGFR (red) and intrinsic fluorescence of wtPLC-γ1 (green) is indicated by arrow. (B) Subcellular fractionation and immunoblotting analysis with anti-GFP, anti-EGFR, anti-Akt and anti-phospho-Akt (p-Akt) antibodies. (C) Quantitation of the results from B and Figure 5.3C. (D) COS-7 cells were transiently transfected with ΔNPH-PLC-γ1 and serum starved for 24 h. The cells were treated with wortmannin (100 nM) for 10 min and then stimulated with EGF for 5 or 30 min. The cells were then processed for immunofluorescence. The colocalization (yellow) of EGFR (red) and intrinsic fluorescence of ΔNPH-PLC-γ1 (green) is indicated by arrow. Size bar = 20 μm.

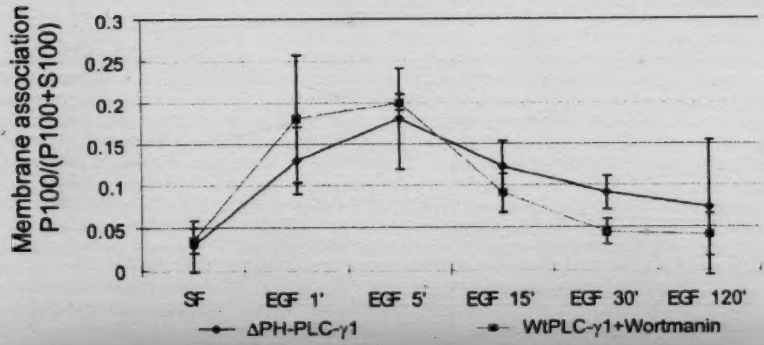
A

SF
EGF 5'
EGF 30'

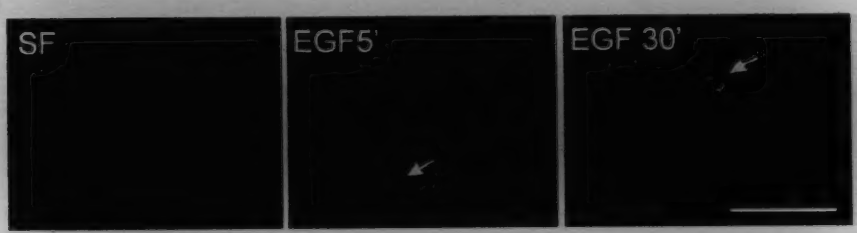
B


P100
S100
EGF - 1' 5' 15' 30' 2h - 1' 5' 15'30' 2h
Total lysates
Anti-GFP
Anti-EGFR
SF EGF5'
Anti-p-Akt
Anti-Akt
Wortmannin
Control

C


Membrane association
P100/(P100+S100)
0.3
0.25
0.2
0.15
0.1
0.05
0
SF
EGF 1'
EGF 5'
EGF 15'
EGF 30'
EGF 120'
ΔPH-PLC-γ1
WtPLC-γ1+Wortmanin

D


SF
EGF5'
EGF 30'

plasma membrane (Figure 5.6A-B). However, after EGF stimulation for 15 and 30 min, only a small portion of mPH-PLC-γ1 was localized to endosomes (Figure 5.6A-B). Subcellular fractionation and immunoblotting further showed that at 5 min, the membrane-bound mPH-PLC-γ1 was at a level similar to that of wtPLC-γ1, however, at 15 and 30 min the membrane-bound mPH-PLC-γ1 was significantly lower than that of wtPLC-γ1. Similar to ΔNPH-PLC-γ1, the point mutation of PH domain did not affect the initial phosphorylation of PLC-γ1 and the interaction between PLC-γ1 and EGFR. However, it did result in the quick decrease of PLC-γ1 phosphorylation following EGF stimulation for 15-30 min (Figure 5.6C). Together, these results suggest that PH domain of PLC-γ1 may function in assisting the stabilization of PLC-γ1 at the membrane, and thus support the sustained activation of PLC-γ1.

5.8 PLC-γ1 activity is not required for EGF-induced translocation of PLC-γ1

I showed above that wtPLC-γ1 was phosphorylated following its translocation to the plasma membrane and remained phosphorylated in endosomes (Figure 5.2). These results raise an interesting question. Is the activation of PLC-γ1 (mediated by its SH2 domains) required for the translocation of PLC-γ1 in response to EGF? To answer this question, I transfected COS7 cells with wtPLC-γ1 and treated the cells with U73122 to inhibit PLC-γ1 activity. I then examined the effects on EGF-induced translocation of wtPLC-γ1. Indirect immunofluorescence showed that inhibition of PLC-γ1 activity did not alter the translocation pattern of wtPLC-γ1 (Figure 5.7A). In the presence of U73122, wtPLC-γ1 was localized to the cytosol without EGF stimulation and translocated to the plasma

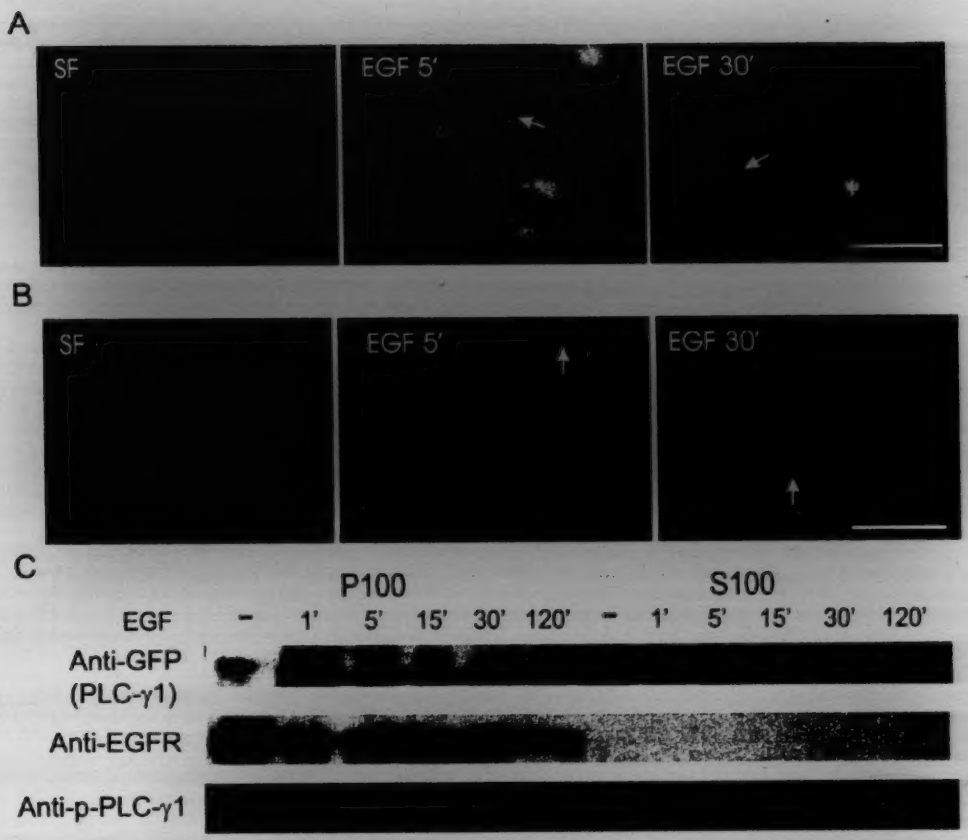


Figure 5.6 EGF-induced translocation of mutant PLC-γ1 with point mutations at the N terminal PH domain (mPH-PLC-γ1). COS-7 cells were transiently transfected with mPH-PLC-γ1 and serum starved for 24 h. (A) The real time images of mPH-PLC-γ1 before and after EGF stimulation. The membrane and endosome localization are indicated by the arrow. (B) Indirect immunofluorescence. The colocalization (yellow) of phospho-EGFR (red) and intrinsic fluorescence of various PLC-γ1 deletion mutants (green) is indicated by the arrow. Size bar = 20 μm. (C) The total cell homogenates were subcellularly fractionated into membrane (P100) and cytosol (S100) and immunoblotted with anti-GFP, anti-EGFR and anti-p-PLC-γ1 antibodies.

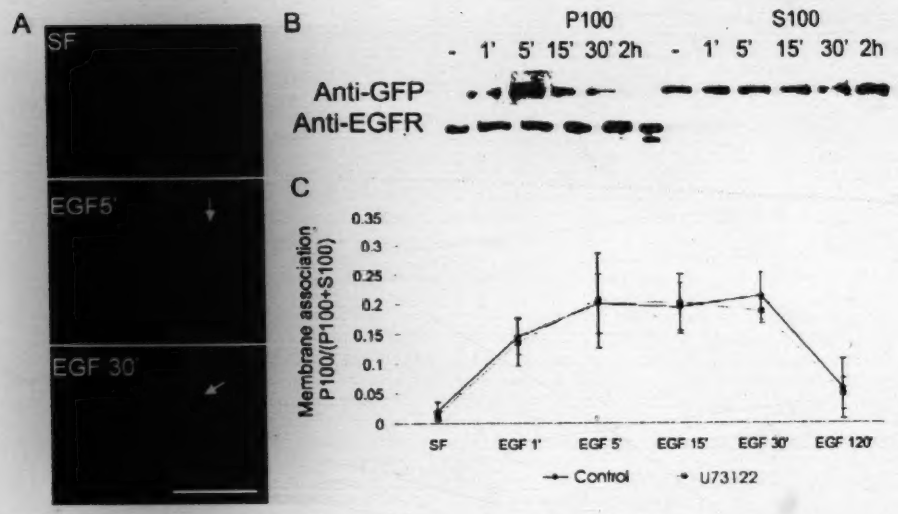


Figure 5.7 Inhibition of PLC-γ1 activity by U73122 and the effect on the translocation of PLC-γ1. COS-7 cells were transiently transfected with wtPLC-γ1 and serum starved for 24 h. The cells were treated with U73122 for 30 min and then stimulated with EGF for indicated times. (A) The cells were analyzed by indirect immunofluorescence. The colocalization (yellow) of EGFR (red) and intrinsic fluorescence of wtPLC-γ1 (green) is indicated by arrow. (B) The cells were subcellularly fractionated and immunoblotted with anti-GFP and anti-EGFR antibodies. (C) Quantitation of the results from E and Figure 5.2D. Size bar = 20 μm.

membrane following EGF stimulation for 5 min. wtPLC-γ1 then translocated to endosomes 30 min after addition of EGF. This finding was further supported by subcellular fractionation and immunoblotting (Figure 5.7B-C). Together, these results suggest that the activation of PLC-γ1 is not required for its translocation.

5.9 Summary

In summary, I provided original evidence demonstrating that 1) endogenous PLC-γ1, similar to YFP-tagged PLC-γ1, translocated to endosomes following its initial translocation from cytosol to the plasma membrane in response to EGF, 2) PLC-γ1 remained phosphorylated in endosomes, however, the PLC-γ1 activity was not required for its translocation, which suggests a signaling role for PLC-γ1 in endosomes, 3) the PH domain was not required for the initial translocation of PLC-γ1 from cytosol to the plasma membrane; however, it stabilizes PLC-γ1 in the membrane at later time points, 4) the function of the PLC-γ1 PH domain in stabilizing PLC-γ1 membrane association is very important in maintaining the activity of PLC-γ1, and 5) the role of PH domain in PLC-γ1 membrane association and activation is dependent on PI3K activity. I conclude that the PLC-γ1 SH2 and PH domains coordinate to determine EGF-induced translocation and activation of PLC-γ1.

Chapter 6

Akt Binds to and Phosphorylates Phospholipase C-γ1 in Response to EGF

The majority of the information presented in this chapeter has been published in Wang, Y., J.Wu and Z.Wang. 2006. Akt Binds to and Phosphorylates Phospholipase C-γ1 in Response to EGF. *Mol. Biol. Cell.* 17:2267-2277. It has been adapted into this Thesis with permission.

6.1 Overview

Both PLC-γ1 and Akt are signaling proteins that play significant roles in the intracellular signaling mechanism utilized by RTKs including EGFR. It is well established that activated EGFR directly stimulates PLC-γ1 by phosphorylating its tyrosine residues and indirectly stimulates Akt by activating PI3K (Carpenter and Ji, 1999). Both PLC-γ1 and PI3K-Akt pathways play significant roles in EGFR-mediated cell signaling. While both PLC-γ1 and PI3K-Akt play distinct roles in cell signaling, the two pathways have been shown to interact with each other. For example, PLC-γ1 and PI3K are both involved in lipid metabolism and use the same substrate (PtdIns(4,5)P_2) (Carpenter and Ji, 1999). Both PLC-γ1 and PI3K-Akt pathways are involved in cell mitogenesis and motility (Chen *et al.*, 1994). It is known that PLC-γ1 may regulate Ras activity through the activation of rasGRP and activated Ras then stimulates the activation of PI3K (Ehrhardt *et al.*, 2004). I have recently shown that PI3K may affect the translocation of PLC-γ1 by generating PtdIns(3,4,5)P_3 (Wang and Wang, 2003). However, it is not known whether PLC-γ1 directly binds to Akt. To gain insight into the molecular interactions between PLC-γ1 pathway and Akt pathways, I determined whether and how PLC-γ1 directly interacts with Akt.

6.2 Co-localization of PLC-γ1 and Akt after EGF stimulation.

Our laboratory has shown that PLC-γ1 translocates to the plasma membrane following EGF stimulation (Wang and Wang, 2003). It has also been reported that Akt translocates to the leading edge of the plasma membrane in response to several growth

factors (Carvalho *et al.*, 2000). However, it is not clear whether PLC-γ1 and Akt co-localize in the plasma membrane following EGF stimulation. I first examine the co-localization of PLC-γ1 and Akt by indirect immunofluorescence. As shown in Figure 6.1A, both PLC-γ1 and Akt were cytosolic without EGF stimulation, and subsequently translocated to the plasma membrane and co-localized together after EGF stimulation for 5 min. Following EGF stimulation for 30 min, some PLC-γ1 was localized to the intracellular vesicles, whereas a significant portion of PLC-γ1 still co-localized with Akt in the plasma membrane (Figure 6.1A).

I further studied the co-localization of PLC-γ1 and Akt by subcellular fractionation. COS-7 cells stimulated with EGF for various perionds of time were cleared of nuclei and subcellularly fractionated into cytosol (Cyt), plasma membrane (PM) and endosomes (EN). Early endosome antigen 1 (EEA1), a protein present in both cytosol and endosomes, was used as a marker for endosomes. EGFR itself is also a marker for both the plasma membrane and endosome as it stays at the plasma membrane without EGF stimulation and internalizes to endosome following EGF stimulation. As shown in Figure 6.1B, both PLC-γ1 and Akt were found mostly in the cytosol before EGF stimulation. After EGF stimulation for 5 min, both PLC-γ1 and Akt were detected in the PM fraction. After EGF stimulation for 30 min, PLC-γ1 was detected in both PM and EN fractions, whereas Akt was only detected in PM fraction. Together, these data indicated that PLC-γ1 and Akt were co-localized to the plasma membrane in response to EGF.

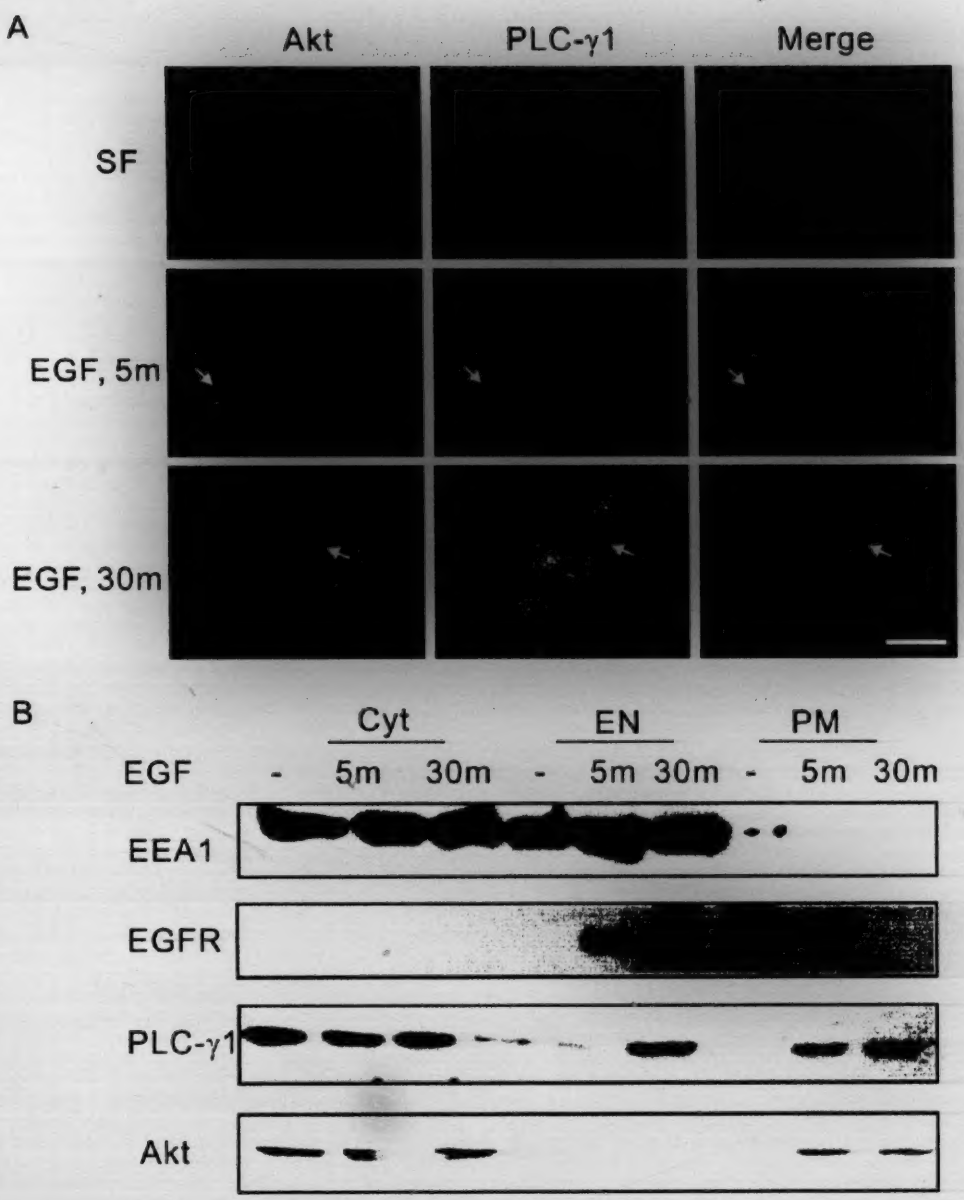


Figure 6.1 Colocalization of PLC-γ1and Akt in COS-7 cells. (A) COS-7 cells were either nontreated or treated with 100 ng/ml EGF for indicated times. The cells were then fixed and stained with anti-PLC-γ1and anti-Akt antibodies. Colocalization (yellow) of Akt (green) and PLC-γ1 (red) is indicated by arrow. Bar, 20 μM. (B) Serum-starved COS-7 cells were either nontreated or treated with 100 ng/ml EGF for 5 or 30 min. The total cell homogenates were subcellularly fractionated into PM, EN, and Cyt. Ten percent protein of each fraction was loaded to 8% SDS-PAGE and immunoblotted with antibodies to Akt, PLC-γ1, EEA1, and EGFR.

6.3 PLC-γ1 forms an EGF-dependent association with Akt

The EGF-dependent co-localization of PLC-γ1 and Akt suggests that they may actually interact each other. Co-immunoprecipitation experiments were performed to examine this possibility. Total cell lysates of COS-7 cells were first immunoprecipitated with an antibody to Akt. Immunoblotting of the immunoprecipitates with anti-PLC-γ1 antibody showed that EGF stimulated strong association between Akt and PLC-γ1 (Figure 6.2A). Moreover, immunoprecipitation of COS-7 cell lysates with anti-PLC-γ1 antibody followed by immunoblotting with anti-Akt antibody further showed EGF-dependent association between PLC-γ1 and Akt (Figure 6.2A). Non-specific mouse IgG and rabbit IgG were used for pre-immune control, and no association was found. A time course experiment was then performed to determine the timing of the interaction. As shown in Figure 2B, the association between PLC-γ1 and Akt started at 5 min and reach the highest point at 15-30 min and remained at a high level for up to 60 min following initial stimulation. After a prolonged stimulation with EGF for 120 min, the association dropped to the background level. Inhibition of PLC-γ1 activity by U73122 did not significantly affect the association between PLC-γ1 and Akt (Figure 6.2B).

To determine the location of the interaction, I further performed the immunoprecipitation experiments after subcellular fractionation. EGF stimulated COS-7 cells were cleared of nuclei and subcellularly fractionated into cytosol (Cyt) and membrane (Mem) fraction. The lysates were immunoprecipitated with an antibody to Akt. Immunoblotting of the immunoprecipitates with anti-PLC-γ1 antibody showed that EGF

stimulated strong association between Akt and PLC-γ1 mostly occurred in membrane (Figure 6.2C). I also observed a basal interaction between PLC-γ1 and Akt in cytosol, which is independent of EGF stimulation (Figure 6.2C)

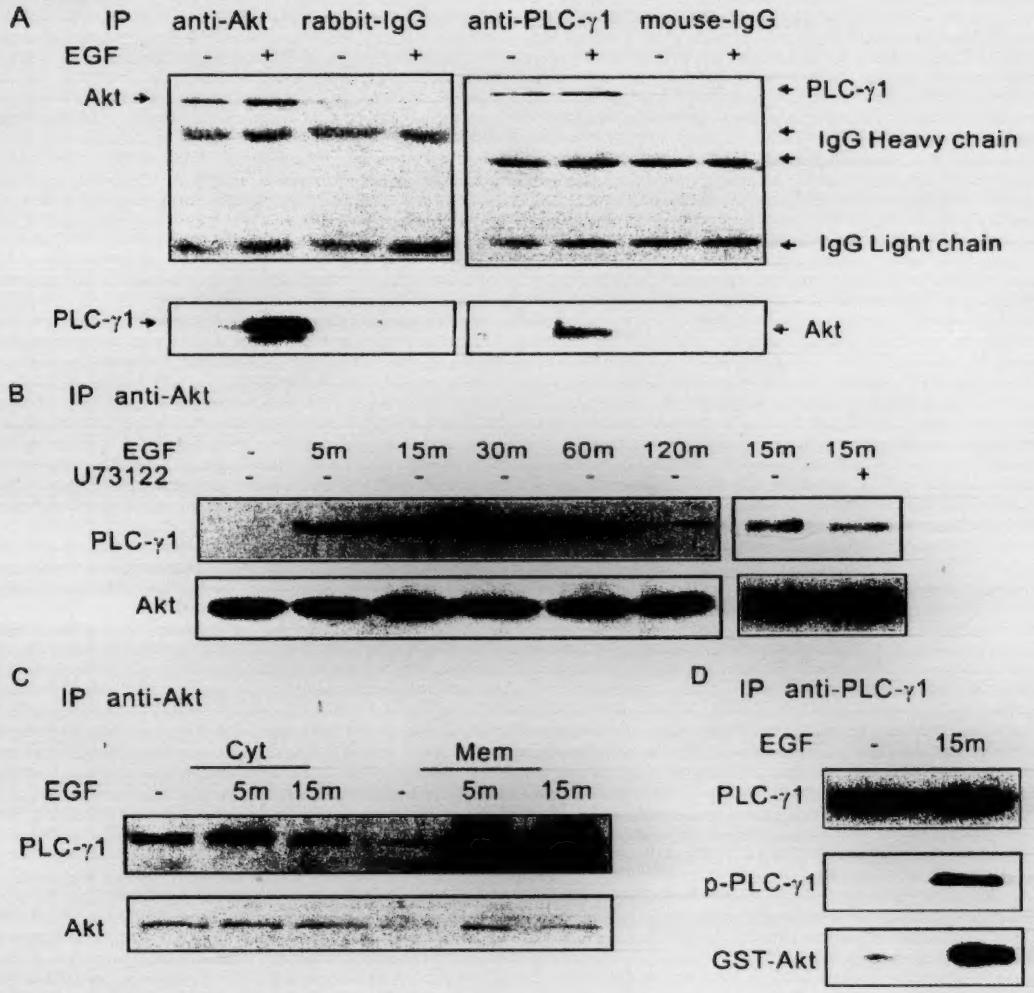
To further examine the association between PLC-γ1 and Akt, I immunoprecipitated PLC-γ1 from COS-7 cells with or without EGF stimulation. As expected, PLC-γ1 was not tyrosine phosphorylated in the absence of EGF, but was strongly tyrosine phosphorylated following EGF stimulation. I then incubated the immunoprecipitates with purified GST-Akt. I showed that only phosphorylated PLC-γ1 bound to GST-Akt (Figure 6.2D).

6.4 SH3 domain of PLC-γ1 is required for the association with Akt

To determine which domain of PLC-γ1 is involved in the association, the binding affinity of each PLC-γ1 domain with Akt was determined *in vitro* using a GST-pull down method. As shown in Figure 6.3A, PLC-γ1 SH3 domain and a region including the SH3 and both SH2 domains (PLC-γ1 SH2-SH2-SH3) were able to bind Akt. PLC-γ1 PH domain, NSH2 domain, and CSH2 domain, in contrast, did not bind to Akt. Interestingly, this binding was constitutive and not EGF dependent. Even treatment with wortmannin, an inhibitor of PI3K, did not abolish the association.

To further determine the role of PLC-γ1 SH3 domain in mediating the interaction between PLC-γ1 and Akt, COS-7 cells were transfected with a mutant PLC-γ1 with the deletion of SH3 domain (ΔSH3-PLC-γ1). I showed by immunoprecipitation that the deletion of SH3 domain completely blocked the association between PLC-γ1 and Akt

Figure 6.2. The EGF-induced association of PLC-γ1 and Akt. (A) COS-7 cells were either not treated or treated with 100 ng/ml EGF for 30 min. The cell lysates were immunoprecipitated with either anti-Akt or anti-PLC-γ1 antibodies and then blotted with anti-PLC-γ1 and anti-Akt antibodies. In controls, the primary antibodies were replaced by normal rabbit IgG and mouse IgG. (B) Serum-starved COS-7 cells were stimulated with 100 ng/ml EGF for 5, 15, 30, 60, or 120 min. Alternatively, serum-starved COS-7 cells were pretreated by 10 μM U73122 for 10 min and then treated with 100 ng/ml EGF for 15 min. The cell lysates were then immunoprecipitated with anti-Akt antibodies, followed by blotting with anti-PLC-γ1 antibody. (C) Serum-starved COS-7 cells were either nontreated or treated with 100 ng/ml EGF for 5 or 30 min. The total cell homogenates were subcellularly fractionated into Mem and Cyt. The proteins of each fraction were immunoprecipitated with antibody to Akt, and the immunoprecipitates were immunoblotted with anti-PLC-γ1and anti-Akt antibodies. (D) Serum-starved COS-7 cells were either nontreated or treated with 100 ng/ml EGF for 15 min, and the cell lysates were then immunoprecipitated with anti-PLC-γ1 antibodies followed by goat anti-mouse IgG conjugated with agarose. After washing three times, GST-Akt was added and incubated for 30 min. The final precipitates were washed and blotted with GST antibody.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PLC-γ1
Akt
D
IP anti-PLC-γ1
EGF - 15m
PLC-γ1
p-PLC-γ1
GST-Akt

(Figure 6.3B). As a control, deletion of PH domain (ΔPH-PLC-γ1) had no effect on EGF-induced association between PLC-γ1 and Akt. These results indicate that the SH3 domain of PLC-γ1 is required for the association between PLC-γ1 and Akt.

To further determine whether the binding between PLC-γ1 and Akt is direct, I performed *in vitro* binding assay by using purified Akt and GST-PLC-γ1SH3 domain. I showed that GST-PLC-γ1 SH3 domain directly bound to Akt *in vitro*, however, GST itself did not bind to Akt. These data indicate that PLC-γ1 directly binds to Akt *in vitro* (Figure 6.3C).

6.5 Proline rich motifs of Akt are required for the association.

Since SH3 domain binds to the P*XX*P motif and Akt contains two P*XX*P motifs (^{424}PFKP^{427} and ^{467}PHFP^{470}) within its C terminus, I next determined the PLC-γ1 binding sites of Akt. Akt-P424A (P424A), Akt-P467A (P467A) and Akt-P424A/P467A (P/P) were created by mutating prolines to alanines. COS-7 cells were transfected with wild type and mutant Akt constructs. The expression level of each construct was normalized to wild type (Figure 6.4A). Immunoprecipitation assay showed that mutation of either P424 or P467 significantly reduced but did not completely abolish the Akt interaction with PLC-γ1, and mutations of both P424 and P467 fully blocked the interaction, indicating that both proline-rich motifs are involved in binding to the SH3 domain of PLC-γ1 (Figure 6.4A). Interestingly, similar to wild type Akt, the mutant P/P translocated from cytosol to the plasma membrane in response to EGF (Figure 6.4B).

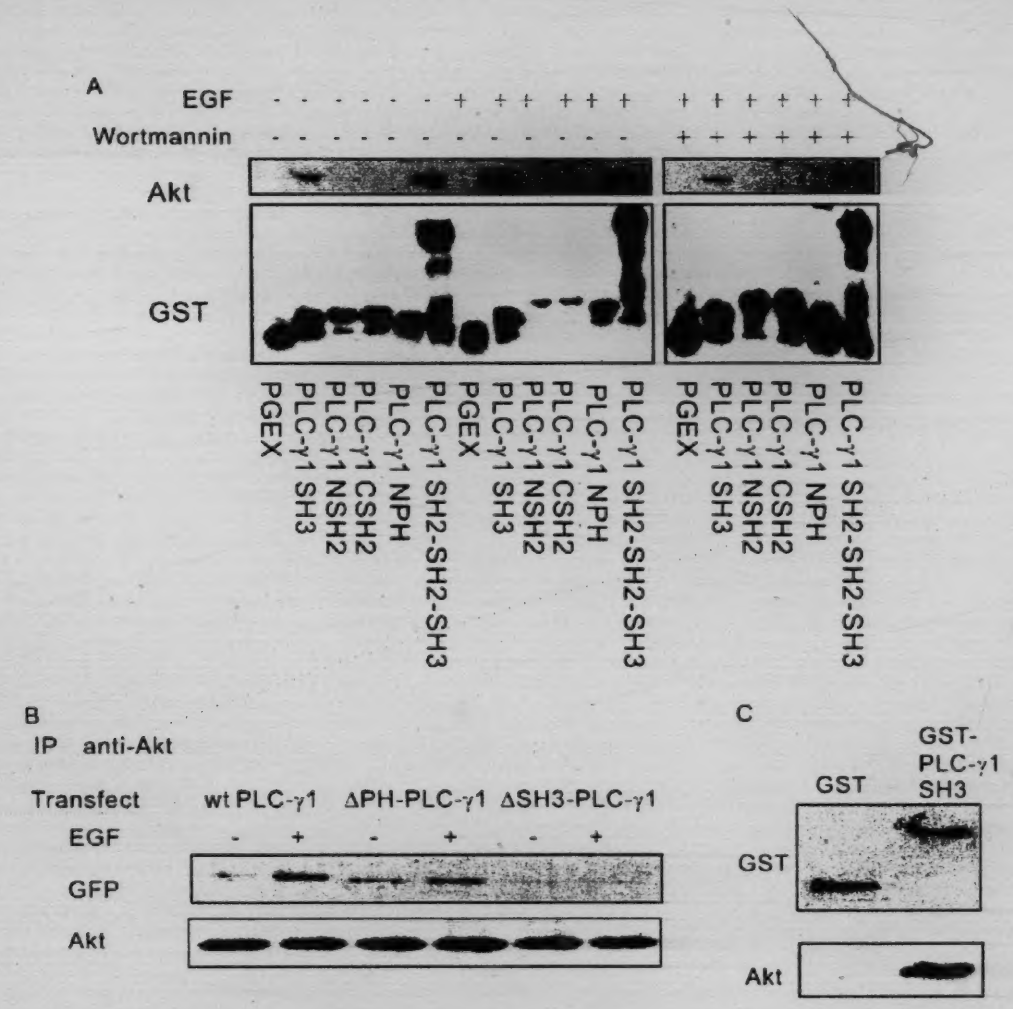


Figure 6.3 SH3 domain of PLC-γ1 is required for the association. (A) The association of Akt with various GST fusion PLC-γ1 domains by GST pull-down. COS-7 cells were not treated, treated with 100 ng/ml EGF for 30 min, or pretreated with 100 nM wortmannin for 30 min followed by 100 ng/ml EGF for 30 min. The cell lysates were incubated with various GST fusion PLC-γ1 proteins bound to glutathione-agarose beads. Bound proteins were analyzed by immunoblotting with anti-Akt antibody. Immunoblotting with anti-GST antibody was used as loading controls. (B) COS-7 cells were transfected with wild type, ΔPH-PLC-γ1, and ΔSH3-PLC-γ1. The cells were either not treated or treated with 100 ng/ml EGF for 30 min. The cell lysates were subjected to immunoprecipitation with monoclonal anti-Akt antibody, and the resulting immunoprecipitates were subjected to immunoblotting with a polyclonal anti-GFP antibody, which reacts to both GFP and YFP. (C) *In vitro* binding assay. GST and GST-PLC-γ1 SH3 beads were incubated with purified Akt for 30 min. After washing three times, the final precipitates were blotted with antibodies to GST and Akt.

6.6 Phosphorylation of Y783 is essential for EGF-mediated association between PLC-γ1 and Akt.

Our immunoprecipitation experiments suggest that PLC-γ1 can form a complex with Akt through its SH3 domain in an EGF dependent manner, whereas our GST pull down experiment suggests that the association between PLC-γ1 SH3 domain and Akt is constitutive and not dependent on EGF. This discrepancy of SH3 domain regulation between *in vivo* and *in vitro* experiments has been documented previously (Kim *et al.*, 2000), but the mechanism remains unclear. I propose that *in vivo* the full length PLC-γ1 adopts a conformation that restricts the interaction of its SH3 domain with Akt. EGF induces the tyrosine phosphorylation of PLC-γ1 that releases the restriction of the SH3 domain and permits the association.

Indeed, the two major phosphorylated tyrosine residues of PLC-γ1, Y771 and Y783 reside between the PLC-γ1 C-SH2 and SH3 domain. To test our hypothesis, I generated three mutants: a mutant with the mutation of its Y771 to phenylalanine (Y771F), a mutant with the mutation of its Y783 to phenylalanine (Y783F) and a PLC-γ1 double mutant with the mutation of its both Y771 and Y783 to the phenylalanines (Y/Y). All the mutants were tagged with YFP at the C-terminus. COS-7 cells were transfected with YFP tagged Y771F, Y783F, Y/Y or wild type PLC-γ1. Cells were stimulated with EGF and the cell lysates were immunoprecipitated with an antibody to Akt. Immunoblotting with antibodies to Akt and GFP showed that Y783F and Y/Y did not bind to Akt, whereas Y771F and wild type PLC-γ1 bind to Akt (Figure 6.5). These results

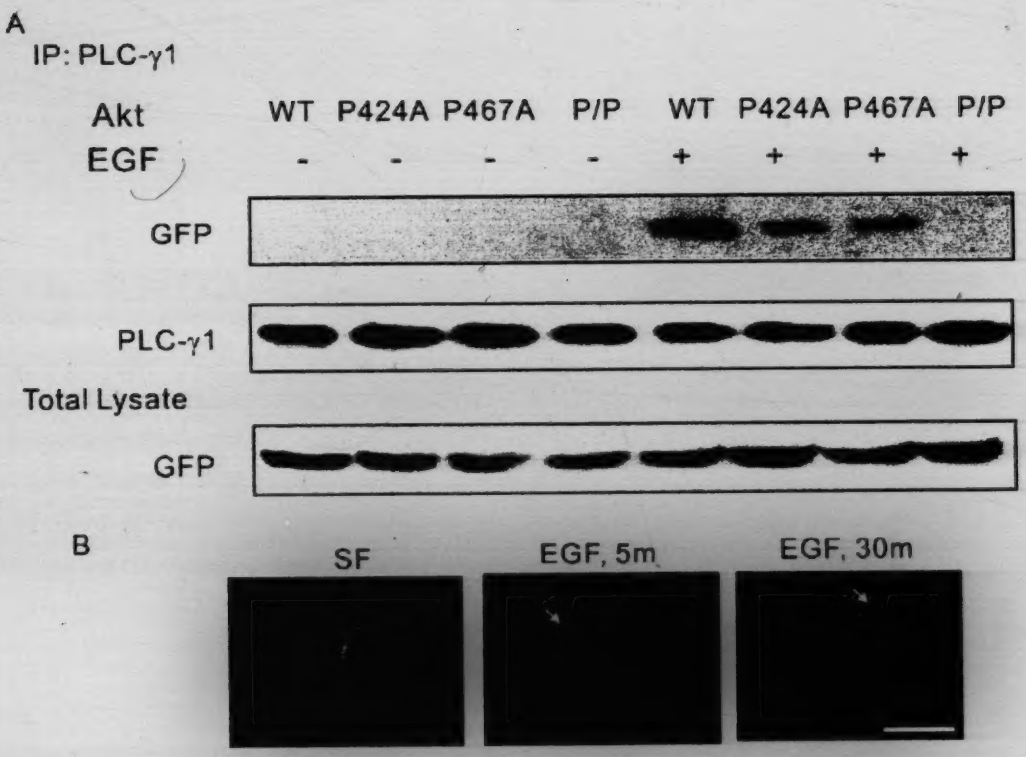


Figure 6.4 Both P424 and P467 of Akt are required for the association of PLC-γ1 and Akt. (A) COS-7 cells were transfected with GFP-Akt or GFP-tagged Akt mutants P424A, P467A, and P/P. The cells were either not treated or treated with 100 ng/ml EGF for 30 min. After treatment, the cell lysates were immunoprecipitated with anti-PLC-γ1 antibody, and the resulting immunoprecipitates were immunoblotted with a polyclonal anti-GFP antibody. The PLC-γ1 blot served as the loading control, and the GFP blot of total lysate served as the control of expression. (B) COS-7 cells were transfected with GFP-Akt-P/P. The cells were then fixed and stained with anti-Akt antibody. The localization of Akt was indicated by arrow. Bar, 20 μM.

suggest that phosphorylation of PLC-γ1 Y783 is essential for EGF-induced association of PLC-γ1 and Akt.

6.7 AKT phosphorylates PLC-γ1 S1248

It was shown previously that PLC-γ1 undergoes serine phosphorylation after RTK stimulation (Kim *et al.*, 1989; Rhee *et al.*, 1990). PLC-γ1 S1248 has been identified as the major serine that is phosphorylated by RTK through protein kinase A (PKA), whereas other unidentified PLC-γ1 serines could also be phosphorylated (Park *et al.*, 1992). Interestingly, Akt is a serine/threonine kinase and the minimal substrate consensus sequence for Akt is RXRXXS/T. The amino acid sequences leading to S1248 is RAREGS1248 that just confer to the minimal substrate consensus sequence for Akt. To determine whether PLC-γ1 is a substrate of Akt, I first inhibited Akt activation by treating COS-7 cells with wortmannin. Immunoblotting with an antibody to phosphorylated Akt (pAkt) showed that wortmannin treatment complete blocked EGF-induced Akt phosphorylation. Immunoblotting with antibody to serine phosphorylated PLC-γ1 S1248 (pS1248) showed that wortmannin treatment significantly reduced the phosphorylation of PLC-γ1 S1248 (Figure 6.6A). To further determine the role of Akt in PLC-γ1 serine phosphorylation, I transfected COS-7 cells with wild type Akt or mutant Akt P/P that does not bind to PLC-γ1. Immunoblotting with anti-pS1248 antibody showed that the phosphorylation of PLC-γ1 S1248 was increased by wild type Akt but decreased by mutant Akt P/P (Figure 6.6B and C). These results suggest that

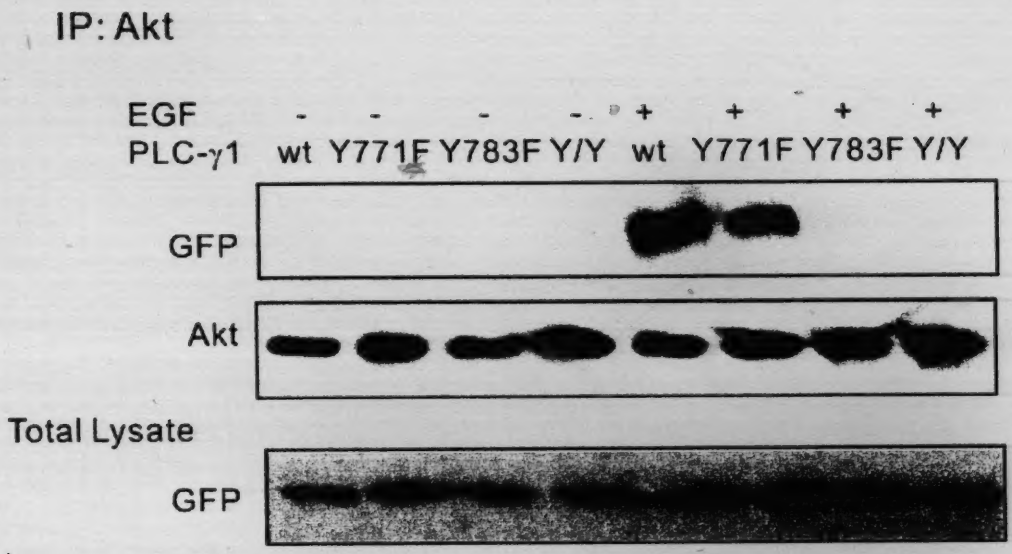


Figure 6.5 Regulation of EGF-induced PLC-γ1 and Akt association by PLC-γ1 Y771 and Y783. COS-7 cells were transfected with YFP-tagged wild-type PLC-γ1 or mutant PLC-γ1 Y771F, Y783F, and Y/Y. The cells were either not treated or treated with 100 ng/ml EGF for 30 min. The cell lysates were then immunoprecipitated with anti-Akt antibody. The resulting immunoprecipitates were subjected to immunoblotting with a polyclonal anti-GFP antibody. The Akt blot served as the loading control, and the GFP blot of total lysate served as the control to show the level of expression.

EGF-stimulated PLC-γ1 S1248 phosphorylation is at least partially dependent on binding to Akt. I also showed that inhibition of PLC-γ1 activity by U73122 had no effect of EGF-induced PLC-γ1 S1248 phosphorylation (Figure 6.6B and C).

Since it has been suggested that PKA could phosphorylate PLC-γ1 S1248 (Park *et al.*, 1992), I further examined the relative contribution of Akt and PKA to EGF induced PLC-γ1 S1248 phosphorylation. I showed that inhibition of PKA by H89 and KT5270 also partially inhibited EGF-induced phosphorylation of PLC-γ1 S1248, whereas inhibition of PKA and Akt simultaneously by treating the cells with wortmannin together with H89 or KT5270 resulted in the complete inhibition of EGF-induced phosphorylation of PLC-γ1 S1248 (Figure 6.6D and E).

6.8 Regulation of EGF-induced cell motility by Akt-PLC-γ1 interaction

PLC-γ1 plays an essential role in EGF-dependent cell motility (Chen *et al.*, 1994). Akt was also shown recently to regulate cell motility by an unidentified mechanism (Firtel and Chung, 2000). Since I showed above that Akt binds to and serine phosphorylates PLC-γ1, I proposed that the Akt-PLC-γ1 interaction is important in mediating EGF-induced cell motility. Wound healing experiments were used to determine cell motility. To test our hypothesis, I first determined whether PLC-γ1 S1248 phosphorylation is important in EGF-induced cell motility. I transfected COS-7 cells with either wild type PLC-γ1 or PLC-γ1 S1248A. Wound healing assays showed that overexpression of wild type PLC-γ1 significantly enhanced EGF-induced cell motility. However, mutation of PLC-γ1 S1248 to alanine largely abolished this enhancement

(Figure 6.7A and B). These results suggest that PLC-γ1 S1248 phosphorylation, accounts for a significant part of cell motility induced by PLC-γ1. It is interesting to note that overexpression of PLC-γ1 S1248 slightly increased cell motility compared with non-transfected cells. This suggests that the role of PLC-γ1 in cell motility is not solely dependent on its S1248 phosphorylation.

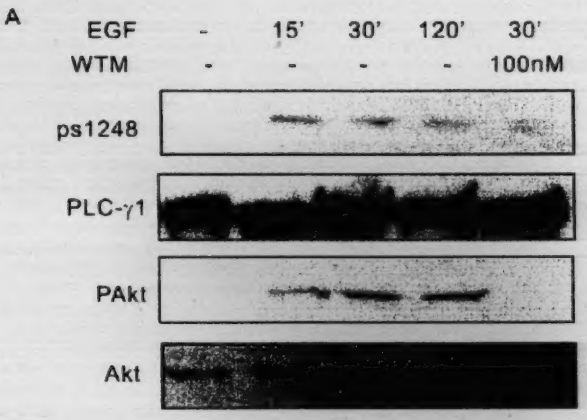
I then examined whether overexpression of wild type Akt in COS-7 cells increased cell motility and whether the effects were dependent on its interaction with PLC-γ1 and dependent on PLC-γ1 activity. When COS-7 cells were transfected with GFP-Akt, wound healing assays showed that overexpression of GFP-Akt increased cell motility by 40% (Figure 6.8AandB). However, further treatment with U73122 to block PLC-γ1 activity abolished this increase. This suggests that the enhancement of Akt on EGF-induced cell motility is dependent on PLC-γ1 activity. Moreover, transfection of COS-7 cells with mutant Akt P/P that does not bind to PLC-γ1 did not increase the cell motility. This suggests that the enhancement of Akt on EGF-induced cell motility is dependent on its interaction with PLC-γ1. Together, these results suggest that Akt-PLC-γ1 interaction plays an important role in EGF-induced cell motility.

6.9 Summary

In summary, I identified a novel interaction between PLC-γ1 and Akt. I demonstrated that the interaction is mediated by the binding of PLC-γ1 SH3 domain to Akt proline rich motifs. Both Akt proline rich motifs interact with PLC-γ1 SH3 domain. I also provide a novel model to depict how the interaction between PLC-γ1 SH3 domain

and Akt proline motifs is dependent on EGF stimulation (Figure 7.3). In this model, the SH3 domain of inactive PLC-γ1 is not accessible to the proline motifs of Akt. Phosphorylation of Y783 by EGFR changed PLC-γ1 conformation by forming an intramolecular association between Y783 and CSH2 domain in PLC-γ1. This conformation change results in the interaction between PLC-γ1 SH3 domain and Akt proline motifs. Furthermore, I showed that the interaction between PLC-γ1 and Akt caused phosphorylation of PLC-γ1 S1248 and plays an important role in EGF-stimulated cell motility.

Figure 6.6 AKT mediates EGF-induced serine phosphorylation of PLC-γ1. (A) COS-7 cells were treated with 100 ng/ml EGF for indicated times with or without 100 nM wortmannin. The total cell lysates were immunoblotted with antibodies to Akt, pAkt, PLC-γ1, and pS1248 of PLC-γ1. (B) COS-7 cells without transfection were treated with 100 ng/ml EGF or EGF and 100 nM wortmannin or U73122. COS-7 cells transfected with GFP-Akt or GFP-tagged mutant Akt P/P were either not treated or treated with EGF. Then, the cell lysates were immunoblotted with anti-pS1248 antibody. Immunoblotting with anti-tubulin antibody was used as loading control and immunoblotting with anti-GFP antibody was used to show the expression level of transfected Akt. (C) Quantification of the data from three independent experiments as described in B. The PLC-γ1 S1248 phosphorylation levels were expressed as the percentage of the level after EGF stimulation. (D) COS-7 cells were treated with 100 ng/ml EGF or EGF with PKA inhibitors H89/KT5270 or with both wortmannin and H89/KT5270. Then, the cell lysates were immunoblotted with anti-pS1248 antibody. (E) Quantification of the data from three independent experiments as described in D. The PLC-γ1 S1248 phosphorylation levels were expressed as the percentage of the level after EGF stimulation.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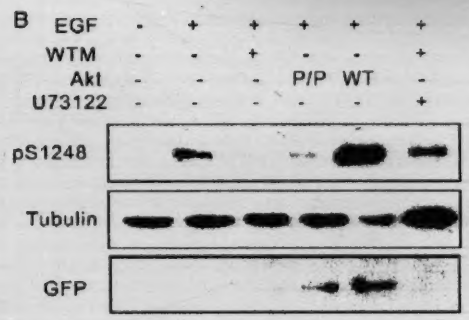
PLC-γ1
PAkt
Akt


B
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Akt - - - P/P WT -
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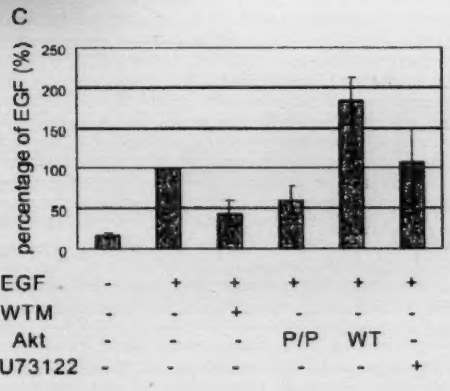
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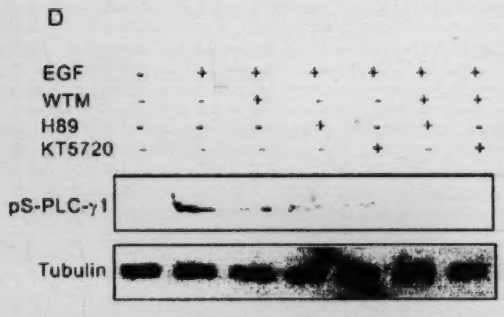
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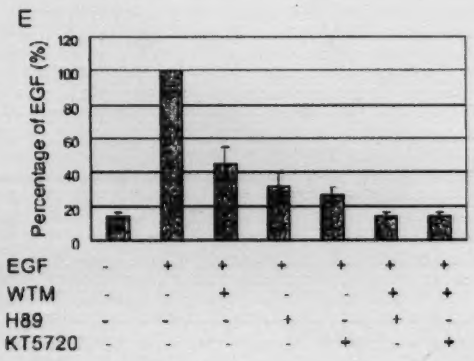
E
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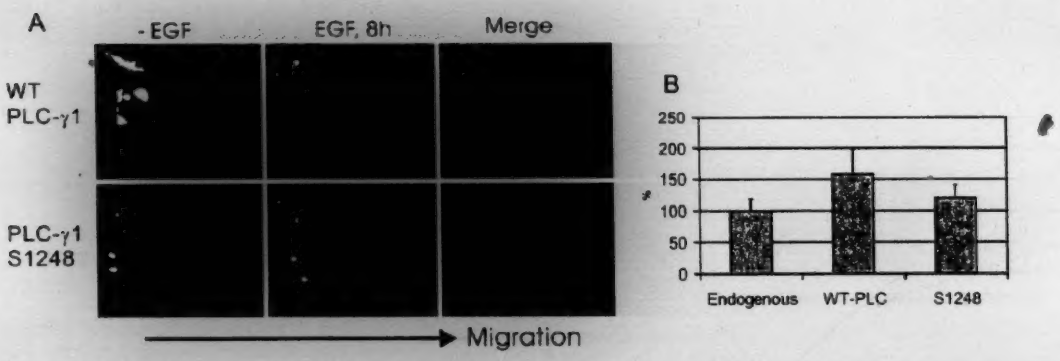

Figure 6.7 Effect of PLC-γ1 S1248 phosphorylation on EGF-induced cell motility. (A) COS-7 cells were transfected with either YFP-tagged wild-type PLC-γ1 or mutant PLC-γ1 S1248A. Confluent monolayers of serum-starved cells were not treated or treated with 100 ng/ml EGF as indicated. Cell motility was examined by a wound healing assay as described in *Materials and Methods*. In the merged image, red shows the cells at 0 h, and green shows the cells at 8 h. All of cells migrated from the left to right as indicated. (B) Quantitation of the data from A. The data were quantitated as described in *Materials and Methods*.

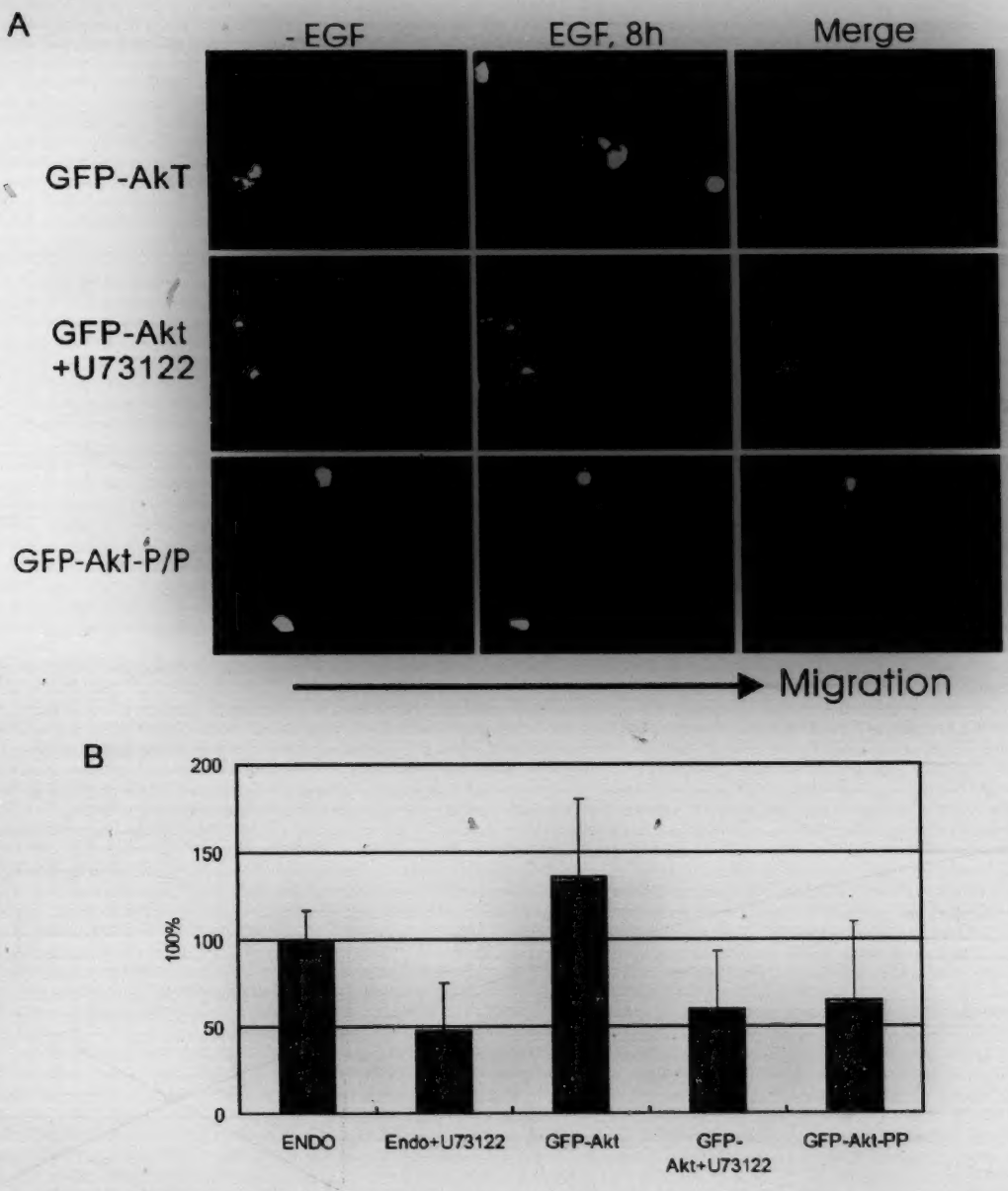

Figure 6.8 Effects of PLC-γ1–Akt interaction and PLC-γ1 activity on EGF-induced cell motility. (A) COS-7 cells were transfected with wild-type GFP-Akt or mutant Akt P/P. Confluent monolayers of serum-starved cells were not treated or treated with 10 μM U73122 and/or 100 ng/ml EGF as indicated. Cell motility was examined by wound healing assay as described in *Materials and Methods*. In the merged image, red shows the cells at 0 h, and the green shows the cells at 8 h. All of cells migrated from the left to right as indicated. (B) Quantitation of the data from A. The data were quantitated as described in **Materials and Methods.**

Chapter 7

Discussion

RTKs and their signaling pathways have profound effects on various cellular processes including cell proliferation, survival, and motility. The understanding of the regulatory mechanisms of RTK signaling will greatly enhance our knowledge about cell signaling networks and the molecular basis underling many human diseases. In this thesis, I studied two aspects of RTK signaling: regulation of RTK signaling by endocytosis and the role of PLC-γ1 in RTK-mediated cell signaling.

7.1 Development of a system to specifically activate EGFR and PDGFR in endosomes

In spite of intensified efforts in understanding cell signaling from endosomes (Clague and Urbe, 2001; Di Guglielmo et al., 1994; Fukazawa et al., 1996; Levkowitz et al., 1998; Wang and Moran, 1996), many questions remain due to the limitations of available approaches. Previous to my thesis work, the most comprehensive approach was to block receptor endocytosis and examine the effects on cell signaling. The most commonly used tools are dominant-negative constructs of the GTPase dynamin and arrestin (Vieira et al., 1996; Ahn et al., 1999; Shen et al., 2001). While such method made significant contributions to our understanding of EGFR signaling and remain powerful tools in theses studies, this approach has serious limitations and insufficiently answered key questions essential for understanding endosomal signaling: 1) whether specific activation of endosome-associated receptors is able to recruit signaling proteins directly into endosomes, 2) whether endosomal signaling is sufficient to stimulate various signaling

pathways leading to cell mitogenesis and survival, and 3) whether endosomal signaling is sufficient to produce a biological outcome. To answer these questions, a novel system needed to be established to activate endosome-associated receptors without activation of the receptors at the cell surface.

As demonstrated in this thesis, I have established such a system to specifically activate EGFR in endosomes without the initial activation at the plasma membrane (Figure 3.1-4). Following treatment with AG1478 to inhibit EGFR kinase activation, addition of EGF resulted in the internalization of inactivated EGF/EGFR complex into endosomes (Figure 3.1-3). In the presence of monensin, 90% of EGFR was internalized and remained in endosomes following EGF treatment for 30 min (Figure 3.2). In contrast, only 70% of EGFR remained in endosome fractions in the absence of monensin, possibly due to the recycling of the inactivated EGF/EGFR complex (Figure 3.3). I did not observe any increase of plasma membrane-associated EGFR following removal of monensin and AG1478, which suggests that washing does not lead to the recycling of EGFR. This result is not unexpected because washing results in the activation of EGFR and activated EGFR is targeted to lysosomes for degradation, instead of recycling back to the plasma membrane. However, in both cases I did not detect any activation of EGFR at the plasma membrane following the activation of endosome-associated EGFR by stripping surface EGF and washing away AG1478 (Figure 3.1-3). The total phosphorylation level of EGFR following its specific activation in endosomes is approximately 50% of that following the standard activation of EGFR at the plasma

membrane (Figure 3.4). Two possible processes may explain the lower phosphorylation level. (1) Some EGF may be dissociated from EGFR in endosomes. (2) AG1478 may not be completely washed away. Nevertheless, our results demonstrate the establishment of a system to specifically activate endosome-associated EGFR without detectable activation of surface EGFR.

Even though PDGFR appears very similar to EGFR in terms of both signaling and trafficking, little is known about the endosomal signaling of PDGFR. In this dissertation, I applied the same approaches from our EGFR study to investigate the endosomal signaling of PDGFR and have successfully generated specific endosomal signaling of PDGFR. I first inhibited PDGFR kinase activity by treating the cells with AG1296, a specific PDGFR kinase inhibitor, and then stimulated cells with PDGF in the presence of monensin. By these treatments, I induced the internalization and accumulation of inactive PDGFR into endosomes (Figure 4.1-2). Removal of AG1296 by washing with serum-free medium led to the activation of endosome-associated PDGFR (Figure 4.1-2). I did not observe any activation of PDGFR associated with the plasma membrane (Figure 4.1-2). The activation (phosphorylation) level of endosome-associated PDGFR is approximately 50% of the control (after standard PDGF activation) (Figure 4.3). The reduced phosphorylation level could result from dissociation of the PDGF-PDGFR complex in endosomes following the removal of AG1296. Our results demonstrate the establishment of a system in which only endosome-associated PDGFR is specifically activated. This system allows us to study PDGFR-mediated signaling from endosomes without

interference from cell-surface PDGFRs.

The EGF and PDGF receptors are first internalized into Rab5-positive endosomes and eventually trafficked to lysosomes for degradation. The differences between standard mitogenic activation and our approach are: (1) EGFR and PDGFR are not activated during their internalization from the plasma membrane into endosomes. (2) These receptors are trapped in endosomes until activation. This system not only allows the generation of pure or nearly pure endosomal signaling of EGFR and PDGFR, but it also does so under a condition very similar to the endosomal signaling of EGFR and PDGFR following its activation at the plasma membrane. The establishment of this system allows us to specifically examine EGFR-mediated signaling and PDGFR mediated signaling from endosomes and to address related questions that cannot be elucidated using previous approaches.

It is important to note that I employed kinase inhibitors to block the kinase activity of RTKs, allowing their internalization into endosomes without activation. Our results are contradictory to recent studies which suggest that EGFR kinase activity is required for EGF-induced EGFR internalization (Lamaze and Schmid, 1995; Sorkina et al., 2002). Several factors may be considered. First, as the inhibition of EGFR kinase results in the recycling of the receptor instead of the degradation of the receptor in lysosomes, it is possible that some studies missed the recycling process and thus concluded that EGFR is not internalized at all. Second, the cell lines and assay methods used in different labs vary greatly. It has been suggested that the requirement for EGFR kinase in EGFR

internalization may vary significantly in different cell lines (Jiang et al., 2003). As our results have clearly shown, in our model system, kinase activity is not involved in the internalization of EGFR.

7.2 Endosome-associated EGFR and PDGFR are able to recruit various signaling proteins to endosomes

Following the establishment of a system to generate specific endosomal RTK signaling, the next question I addressed was whether the activated endosome-associated EGFR could recruit various signaling proteins to endosomes. It has been argued that clathrin coated pits function as nucleation sites for the organization of signaling complexes on the plasma membrane (Burke et al., 2001; Haugh et al., 1999; Kuruvilla et al., 2000; Miller and Lefkowitz, 2001; Slepnev and De Camilli, 2000). However, I showed by co-immunoprecipitation that activation of endosome-associated EGFR and PDGFR resulted in the interaction with various signaling proteins including SHC, Grb2, and the p85α subunit of PI3K (Figure 3.5 and Figure 4.4). Our results indicate that endosomes can also serve as nucleation sites for the organization of signaling complexes. This indicates that the plasma membrane is not a privileged site for protein recruitment and receptor-signal complex formation.

7.3 Endosomal signaling of EGFR and PDGFR are sufficient to stimulate various signaling pathways

Another question I addressed is whether endosomal activation of EGFR and

PDGFR are sufficient to stimulate various signaling pathways. Previous studies using endocytosis inhibition approach suggest that endosomal signaling of RTK is necessary for RTK-mediated ERK activation (Burke et al., 2001; Haugh et al., 1999; Kuruvilla et al., 2000; Miller and Lefkowitz, 2001; Slepnev and De Camilli, 2000). Here I showed that endosomal signaling of EGFR and PDGFR itself is sufficient to activate various signaling proteins including Ras, ERK and Akt (Figure 3.6 and Figure 4.5). I further showed that endosomal signaling of EGFR and PDGFR stimulates Erk activation by a Ras-Raf-MEK-Erk pathway, which is similar to the standard activation of EGFR and PDGFR at the plasma membrane (Figure 3.6 and Figure 4.5). When wortmannin was used to inhibit the function of PI3K, Akt phosphorylation was totally blocked, indicating Akt activation is mediated by PI3K when initiated from an endosomally activated receptor (Figure 3.6 and Figure 4.5). I also detected the phosphorylation of PLC-γ1 from total lysates of cells following the activation of endosomal PDGFR (Figure 4.5), suggesting that PLC-γ1 behaves similarly regardless of the localization of PDGFR's signal origin. Interestingly, PLC-γ1 is only weakly phosphorylated by endosomal signaling of EGFR, suggesting that endosomal signaling of EGFR is not identical to the standard EGFR signaling. In addition, the weak phosphorylation of PLC-γ1 demonstrates that endosomal signaling of EGFR is different from that of PDGFR (Figure 3.6).

7.4 Endosomal signaling of EGFR and PDGFR is sufficient to suppress apoptosis and induce mitogenesis

I determined the physiological relevance of EGFR-mediated signaling from

endosomes. Previous findings have suggested that inhibition of EGFR endocytosis blocks EGF-stimulated ERK activation (Burke et al., 2001; Haugh et al., 1999; Kuruvilla et al., 2000; Miller and Lefkowitz, 2001; Slepnev and De Camilli, 2000). However, in the few cases where biological end points were measured, inhibition of endocytosis did not result in attenuation of biological effects (Vieira et al., 1996; Ceresa et al., 1998). Very recently, it was reported that retrograde support of neuronal survival does not require the retrograde transport (endocytosis) of nerve growth factor (MacInnis and Campenot, 2002). These results argue against a physiological relevance of endosome-originated signals (Di Fiore et al., 1987). Here I showed that endosomal signaling of EGFR and PDGFR itself stimulated the PI3K-Akt pathway, which suggests a role for endosomal EGFR and PDGFR signaling in anti-apoptosis. Indeed, I showed by TUNEL assay that activation of endosome-associated EGFR and PDGFR suppressed apoptosis induced by serum-withdrawal (Figure 3.7 and Figure 4.6). Moreover, this suppression was blocked by treating the cell with wortmannin. I also showed that endosomal signaling of EGFR and PDGFR is sufficient to activate Akt and this activation is abolished by wortmannin. Together, my results indicate that the molecular mechanism by which endosomal signaling of EGFR and PDGFR supports cell survival is through the activation of the PI3K-Akt pathway. This provides original evidence that endosomal signaling is sufficient to produce biological outcomes.

The activation of PI3K-Akt and Ras-Erk-c-Fos/c-Jun pathway by endosome-associated EGFR suggests that the stimulation of endosome-associated EGFR

may play a role in cell proliferation. However, our unpublished results suggested that without the continuous presence of EGF, activation of endosome-associated EGFR itself did not stimulate BrdU incorporation because the EGFR remained activated for less than 2 h. This is consistent with previous finding that EGF needs to be in the medium for more than 8 h to stimulate DNA synthesis. The difficulty in demonstrating mitogenic outcome from endosomal EGFR signals was the inability to maintain the receptor in endosomes for a prolonged period. After 2 h, most of the activated, internalized receptor has been degraded. While it may sustain endosomal EGFR signaling, inhibition of lysosomal trafficking will alter the normal trafficking route of EGFR and thus compromise the physiological relevance of the outcome. Recent insights into growth factor-dependent mitogenesis, however, may provide an opportunity for us to determine the role of endosomal EGFR signaling in cell proliferation. In platelet-derived growth factor (PDGF)-induced fibroblasts, serum-arrested cells can be driven into S phase by using two discontinuous pulses of ligand spaced 8 h apart in place of continuously exposing cells to ligand for 8 h with equal proliferative kinetics. I also showed that two discontinuous pulses derived from endosomal PDGFR was sufficient to drive the majority of quiescent cells into S-phase, with kinetics comparable with either standard two-pulse induction of PDGF, or continual exposure with mitogen for 8 hours (Figure 4.7).

Since the EGFR system is very similar to the PDGF receptor (PDGFR) system, both in signaling pathways and biological outcomes, our laboratory hypothesized that two short pulses of EGFR endosomal signaling are sufficient to stimulate cell proliferation.

Recent studies from our laboratory suggested that two pulses of endosomal EGFR signaling are sufficient to stimulate cell proliferation. It was shown that two pulses of endosomal EGFR signaling engaged cell cycle machinery the same way as the two pulses of standard EGFR signaling. Moreover, two pulses of endosomal EGFR signaling stimulated downstream signaling cascades in a similar way to the two pulses of standard EGFR activation (Pennock and Wang, 2003).

7.5 PLC-γ1 translocates from cytosol to plasma membrane through the coordination between SH2 and PH domain

The PLC-γ1 pathway is the major signaling pathway downstream of RTKs. It has been implicated in many GF-induced cell signaling pathways, including cell proliferation, differentiation, receptor endocytosis, cell motility, membrane ruffle formation and branching tubulogenesis (Wahl and Carpenter, 1991; Kamat and Carpenter, 1997; Wells et al., 1998; Gual et al., 2000; Meyer et al., 2003; Bivona et al., 2003; Chou et al., 2003; Choi et al., 2004). PLC-γ1 translocates among various subcellular compartments following EGF stimulation. Various PLC-γ1 domains including SH2, SH3 and PH domains have been implicated in determining the localization and translocation of PLC-γ1 (Cantley et al., 1991; Bae et al., 1998; Ye et al., 2002; Wang et al., 2001). However, PLC-γ1's translocation pattern and the molecular mechanism of PLC-γ1 translocation are far from fully understood. In this study, I attempted to define the PLC-γ1 translocation pattern and the molecular mechanism of PLC-γ1 translocation by multiple approaches.

First, I determined the translocation pattern of PLC-γ1. While the translocation of PLC-γ1 from cytosol to the plasma membrane has been well established, the subsequent trafficking is still not well defined. GFP tagged PLC-γ1 was co-localized with EGFR to endosomes in transfected A431 cells (Wang et al., 2001). However, it was not clear whether the endosome-association of PLC-γ1 was merely due to the overexpression. The issue was further complicated by another recent report showing that PLC-γ1 translocated to the nucleus following NGF stimulation(Ye et al., 2002). Here I provided convincing evidence that both endogenous PLC-γ1 and transfected wtPLC-γ1 initially translocate from cytosol to the plasma membrane and then translocate into endosomes in response to EGF stimulation (Figure 5.1 and 2). This suggests that the translocation of PLC-γ1 to endosomes is a truly physiological phenomenon. No nuclear localization of either endogenous or YFP tagged PLC-γ1 was observed (Figure 5.1 and 2).

Another original finding of my study is that endosome-associated PLC-γ1 remains phosphorylated following its activation at the plasma membrane (Figure 5.1 and 2) and the activation of PLC-γ1 is not required for its translocation (Figure 5.7). These findings suggest that PLC-γ1 may continue to function in endosomes. However, conflicting results have been reported in this regard. On the one hand, immunogold labeling of PLC-δ1-PH domain revealed high levels of PtdIns(4,5)P_2 in the plasma membrane as well as significant levels in intracellular membranes, including endosomes (Watt et al., 2002). This suggests that PLC-γ1 may continue to function as an enzyme to hydrolyze PtdIns(4,5)P_2 and mediate cell signaling in endosomes. On the other hand, it was reported

that there is very little, if any, PtdIns(4,5)P_2 in endosomes and that PLC translocation to endosomes may in fact serve to terminate signaling rather than to prolong it (Haugh et al., 1999). However, it is still possible that the phospho-PLC-γ1 may play a signaling role independent of its enzymatic activity in endosomes.

I determined the roles of PLC-γ1 individual domains in regulating EGF-induced translocation of PLC-γ1 by using a battery of domain deletion mutants of PLC-γ1 including ΔNSH2-PLC-γ1, ΔCSH2-PLC-γ1, ΔPH-PLC-γ1, and ΔSH3-PLC-γ1. In agreement with previous publications, I showed that the two SH2 domains are required for EGF-induced translocation of PLC-γ1 to the plasma membrane. It has been reported that PLC-γ1 is targeted to the cytoskeleton via its SH3 domain, where it functions to regulate actin polymerization (Bar-Sagi et al., 1993). However, by deleting PLC-γ1 SH3 domain, I did not observe significant change in PLC-γ1 translocation either at the initial step of plasma membrane targeting, or in its subsequent translocation to endosomes (Figure 3). This suggests that the SH3 domain is not involved in the translocation of PLC-γ1.

A major finding of this study is the novel role of the N-terminal PH domain of PLC-γ1 in mediating its translocation. The results regarding the N-terminal PH domain of PLC-γ1 in the translocation of PLC-γ1 have been very controversial. It was reported that overexpression of the PLC-γ1 PH domain prevents PLC-γ1 membrane targeting, and this suggested that the PH domain of PLC-γ1 mediates membrane localization (Falasca et al., 1998). However, two more recent publications demonstrated that the translocation of

PLC-γ1 to the plasma membrane required functional SH2 domains and was not affected by mutations in the PH domain or inhibition of PI3K (Matsuda et al., 2001; Wang et al., 2001). My results suggested that the PH domain was not required for the initial translocation of PLC-γ1 from cytosol to the plasma membrane. However, it functioned at a later time to stabilize PLC-γ1 in the plasma membrane (Figure 5.3). Furthermore, I showed that deletion of the PH domain results in the early decrease of PLC-γ1 phosphorylation (Figure 5.4). This positively correlates with the early dissociation of PLC-γ1 from the membrane. It is likely that in the absence of the PH domain the PLC-γ1 quickly dissociates from the plasma membrane, which leads to the inactivation of PLC-γ1. This suggests that the function of PLC-γ1 PH domain in stabilizing PLC-γ1 membrane association is very important in maintaining the activity of PLC-γ1. To eliminate the possibility that EGF-stimulated translocation of PLC-γ1 is due to the conformational change induced by the PH domain deletion, I generated a mutant PLC-γ1 with a point mutation at its PH domain (mNPH-PLC-γ1). I showed that in COS-7 cells transfected with mPH-PLC-γ1, EGF induced translocation of mNPH-PLC-γ1 shared the same pattern as ΔNPH-PLC-γ1 (Figure 5.6)

The next question addressed in this thesis is how the PH domain stabilizes PLC-γ1 in the plasma membrane only at a late time point, but not at the initial stage. I showed that the late stabilization role of PH domain is dependent on PI3K activity (Figure 5.5). In response to EGF, EGFR interacts with PI3K and PLC-γ1, and stimulates the activation of both PI3K and PLC-γ1. The activated PI3K then catalyzes the formation of

PtdIns(3,4,5)P_3 that provides a binding site for the PLC-γ1 PH domain. Indeed, PI3K-dependent translocation of PH domain occurs at 5 min of EGF stimulation (Falasca et al., 1998), which is later than EGF-induced translocation of PLC-γ1 that occurs at 1 min of EGF addition (Figure 5-2) (Todderud et al., 1990; Kim et al., 1990). By showing the distinct roles of SH2 and PH domains in EGF-induced translocation of PLC-γ1, our results thus clear the controversy over this issue.

7.6 Akt binds to and phosphorylates PLC-γ1 in response to EGF

In Chapter 6 of this dissertation, I identified a novel interaction between PLC-γ1 and Akt. I showed by immunofluorescence and subcellular fractionation that PLC-γ1 and Akt were co-localized to the plasma membrane following EGF stimulation (Figure 6.1). I also showed by immunoprecipitation, GST-fusion protein pull-down experiments and in vitro binding experiments that PLC-γ1 and Akt directly bind to each other (Figure 6.2 and 3). It is well established that activated EGFR directly stimulates PLC-γ1 by phosphorylating its tyrosine residues and indirectly stimulates Akt by activating PI3K. Both PLC-γ1 and PI3K-Akt pathways play significant roles in EGFR-mediated cell signaling. While both PLC-γ1 and PI3K-Akt play distinct role in cell signaling, the two pathways have been shown to interact each other. For example, PLC-γ1 and PI3K are both involved in lipid metabolism and use the same substrate (PtdIns(4,5)P_2) (Carpenter and Ji, 1999). Both PLC-γ1 and PI3K-Akt pathways are involved in cell mitogenesis and motility (Chen et al., 1994). It is also known that PLC-γ1 may regulate Ras activity through the activation of RasGRP and activated Ras then stimulates the activation of

PI3K (Ehrhardt et al., 2004) (Rodriguez-Viciana et al., 1994). Our previous results suggest that PI3K might affect the translocation of PLC-γ1 by generating PtdIns(3,4,5)P_3. So far my data have demonstrated that the interaction between the PLC-γ1 and PI3K-Akt pathways involve exclusive interaction between PLC-γ1 and PI3K. Here I demonstrate that PLC-γ1 and Akt directly bind each other, providing another layer of interaction between these two pathways.

I further demonstrated that the interaction between PLC-γ1 and Akt is mediated by PLC-γ1 SH3 domain (Figure 6.3) and the proline rich motifs of Akt (Figure 6.4). PLC-γ1 is a lipase with multiple domains, which allows the interaction of PLC-γ1 with various proteins to form large protein complexes. It is clear that PLC-γ1 SH2 domains interact with the phosphotyrosine of RTKs. However, the function of PLC-γ1 SH3 and PH domains are still not clear. Interestingly, most newly identified interactions between PLC-γ1 and other proteins are mediated by its SH3 domains. It was recently reported that PLC-γ1 directly interacts with various cellular proteins including PLD_2 (Jang et al., 2003), PIKE (Ye et al., 2002), dynamin (Choi et al., 2004), Emt (Perez-Villar and Kanner, 1999), and SOS (Kim et al., 2000) through its SH3 domain. My studies append Akt to this list of interacting partners.

Akt contains two proline rich motifs (^{424}PFKP^{427} and ^{467}PHFP^{470}) within its C terminus. I showed that point mutation of either P424 or P467 to alanine significantly reduced but not completely abolished the interaction between PLC-γ1 and Akt, whereas a mutant Akt with both proline mutated to Alanine (P/P) did not bind to PLC-γ1 at all

(Figure 6.4). This suggests that both proline motifs of Akt are involved in the interaction with PLC-γ1.

In contrast to the interaction between the SH2 domain and phosphotyrosine motif, the interaction between the SH3 domain and proline rich motifs does not require the post-translational modification of either the SH3 domain or proline motifs. Indeed, I showed here by *in vitro* binding assay that the interaction between PLC-γ1 SH3 domain and Akt is constitutive (Figure 6.3). However, these data seem at odds with our *in vivo* data showing that the interaction between the full-length PLC-γ1 and Akt is dependent on EGF stimulation. Similar observations have been made for the interactions between PLC-γ1 and other proteins, such as PLD_2 (Jang et al., 2003), PIKE (Ye et al., 2002), dynamin (Choi et al., 2004), and Emt (Perez-Villar and Kanner, 1999).

In this thesis, I provide a novel model to address this discrepancy. I proposed *that in vivo* the full length PLC-γ1 adopted a conformation that restricts the interaction of its SH3 domain with Akt. Phosphorylation of PLC-γ1 Y771 and/or Y783 by EGF stimulation releases this restriction on the SH3 domain (Figure 7.3). Our model is supported by the following evidence. First, Y771 and Y783 are the major tyrosine residues phosphorylated by EGFR and locate between PLC-γ1 SH3 and CSH2 domains. Thus, it is highly possible that the phosphorylation of Y771 and/or Y783 may have major effects on the conformation of PLC-γ1. Second, it was recently reported that in response to EGF, the phosphorylated Y783 forms an intramolecular interaction with the CSH2 domain in the activated PLC-γ1 (Poulin et al., 2005). Third, I showed in this study that

double mutation of Y771 and Y783 or single mutation of Y783 blocked EGF-induced association between PLC-γ1 and Akt (Figure 6.5).

I also examined the role of PLC-γ1-Akt interaction in EGF-induced cell signalling. Akt is a serine/threonine kinase. The amino acid sequences leading to S1248 is (RAREGS1248) which conforms to the minimal substrate consensus sequence for Akt (RXRXXS/T). Indeed, I showed that the interaction between PLC-γ1 and Akt resulted in the phosphorylation of PLC-γ1 S1248 by Akt (Figure 6.6). Inhibition of Akt phosphorylation by wortmannin or disruption of PLC-γ1 and Akt interaction by mutation partially blocked EGF-induced phosphorylation of PLC-γ1 S1248 (Figure 6B), which suggests that EGF-induced PLC-γ1 S1248 phosphorylation is partially dependent on binding to Akt. Since it has been suggested that PKA could phosphorylate PLC-γ1 S1248 (Park et al., 1992), I further examined the relative contribution of Akt and PKA on EGF-induced PLC-γ1 S1248 phosphorylation. Our results indicated that both Akt and PKA partially contributed to EGF-induced PLC-γ1 S1248 phosphorylation (Figure 6.6D and E).

Finally, I showed that the interaction between PLC-γ1 and Akt plays important role in EGF-induced cell motility. Both PLC-γ1 and Akt have been shown to regulate EGF-induced cell motility (Chen et al., 1994). I showed here that the enhancement of EGF-induced cell motility by PLC-γ1 is mostly abolished by mutation of S1248 (Figure 6.7A and B). This indicates that S1248 phosphorylation by Akt is important in mediating EGF-induced cell motility. I further showed that the enhancement of cell motility by Akt

is abolished by blocking the interaction between Akt and PLC-γ1 and by inhibiting PLC-γ1 activity (Figure 6.8 A and B). All these data support an important role for Akt-PLC-γ1 interaction in EGF-induced cell motility.

7.7 Summary, Conclusion and General Model

In this thesis, I studied two aspects of RTK signaling and the regulation: regulation of RTK signaling by endocytosis and the role of PLC-γ1 in RTK-mediated cell signaling. The research presented in this dissertation supports the following conclusion: The coordination of EGFR trafficking and spatial restriction of signal specificity may represent a general mechanism used to regulate signal transduction and modulate signal specificity.

I used both EGFR and PDGFR systems to study the regulation of RTK signaling by endocytosis. I concluded that endosomes can serve as a nucleation site for the formation of signaling complexes and that endosomal EGFR signaling is sufficient to activate the major signaling pathways leading to cell proliferation and anti-apoptosis. Based on our results and the results from other laboratories, I propose a model to describe the relationship between RTK-mediated signal transduction and RTK endocytosis. In this model, qualitatively, the EGFR-mediated signaling from endosomes is the continuation of the EGFR signaling from the plasma membrane. This model clearly demonstrates that eliminating the initial signaling from the plasma membrane does not affect the ability of EGFR to activate major signaling pathways and to provide physiological response. Moreover, following EGF stimulation, activated EGF receptor only remains at the plasma

membrane briefly (5-10 min), but remains at endosomes much longer (1 h). This argues for a more important role for endosome signaling. By this model I can well explain the long enduring controversy regarding signaling from endosomes. If EGFR signaling from endosomes exists and is important, why does inhibition of the internalization of EGFR that eliminates the EGFR signaling from endosomes result in enhanced cell proliferation? My model predicts that if EGFR endocytosis is blocked at any point before its trafficking to lysosome for degradation, it will prolong EGFR-mediated signaling and thus enhance EGF-stimulated cell proliferation (Figure 7.1).

As to EGF-induced translocation of PLC-γ1, I showed that PLC-γ1 SH2 and PH domains coordinate to determine EGF-induced translocation and activation of PLC-γ1. Based on these results and the results for other laboratories, I propose a "recruit and stabilize" model. In this model PLC-γ1 is mainly located in the cytosol without EGF stimulation (Figure 7.2A). Following EGF stimulation for 1 min, PLC-γ1 is recruited to the plasma membrane via the interaction between its SH2 domains and the phosphotyrosine residues of EGFR (Figure 7.2B). This binding leads to activation of PLC-γ1. Simultaneously, EGF activates PI3K to produce PtdIns(3,4,5)P_3. The phosphorylated PLC-γ1 then interacts with PtdIns(3,4,5)P_3 through its PH domain to further stabilize the PLC-γ1 at the plasma membrane (Figure 7.2C). The translocation of PLC-γ1 is accompanied with the translocation of Akt from cytosol to plasma membrane which is mediated by its PH domain and PtdIns(3,4,5)P_3. I demonstrated that there is a direct interaction between PLC-γ1 and Akt and this interaction is mediated by the binding

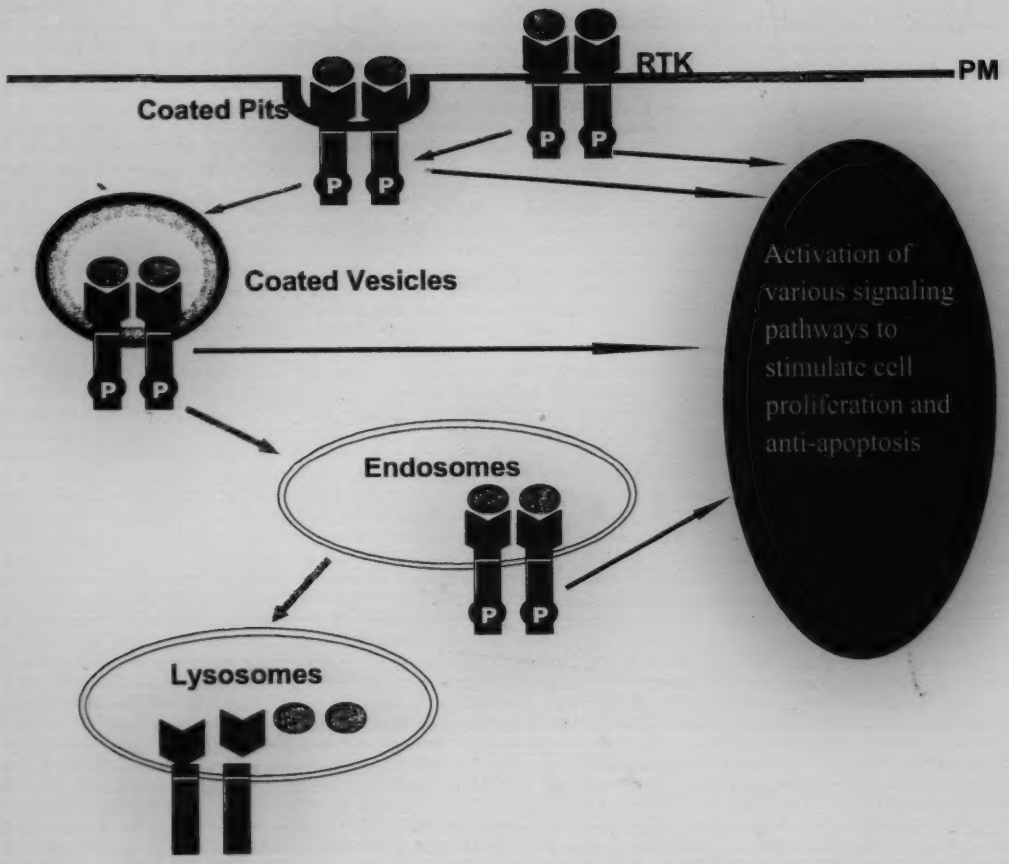

Figure 7.1 Continuous signaling of RTKs from endosomes. Endosomes can serve as a nucleation site for the formation of signaling complexes and endosomal RTK signaling is sufficient to activate the major signaling pathways leading to cell proliferation and anti-apoptosis.

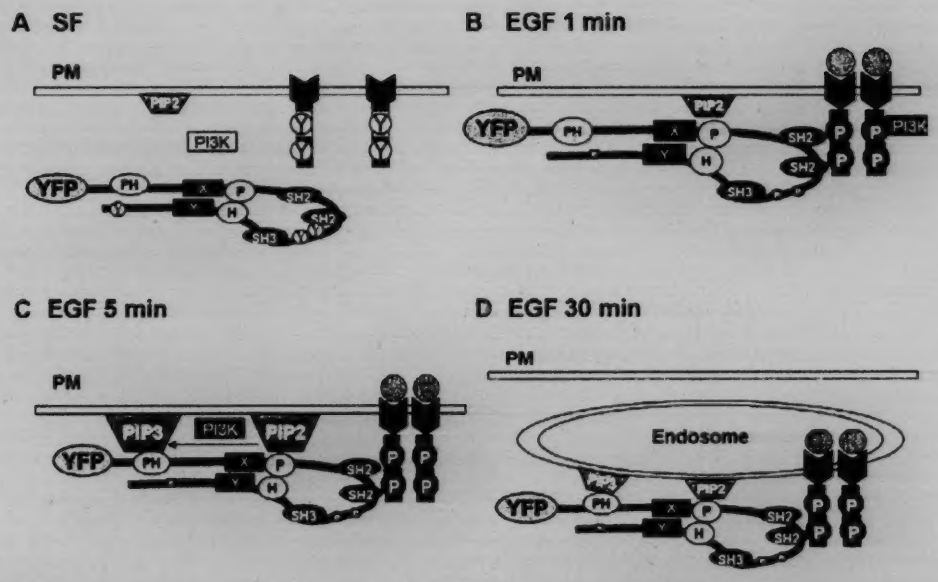

Figure 7.2 The 'recruit and stabilize' model of EGF-induced PLC-γ1 translocation. (A) PLC-γ1 and PI3K mainly locates in cytosol without EGF stimulation. (B) Following EGF stimulation for 1 min, PLC-γ1 is recruited to the plasma membrane via the interaction between its SH2 domains and the phosphotyrosine residues of EGFR. Simultaneously, EGFR recruits and activates PI3K to produce PtdIns(3,4,5)P_3 at plasma membrane. (C) The phosphorylated PLC-γ1 then interacts with PtdIns(3,4,5)P_3 through its PH domain to further stabilize PLC-γ1 at the plasma membrane. (D) PLC-γ1 translocates to endosomes with EGFR.

of PLC-γ1 SH3 domain to Akt proline rich motifs dependent on EGF stimulation (Figure 6.9). Shortly after this, PLC-γ1 translocates to endosomes with EGFR (Figure 7.2D).

I also provide a novel model to explain the EGF dependent association between PLC-γ1 SH3 domain to Akt proline rich motifs (Figure 7.3). In this model, the SH3 domain of inactive PLC-γ1 is not accessible to the proline motifs of Akt. Phosphorylation of Y783 by EGFR changed PLC-γ1 conformation by forming an intra-molecular association between Y783 and CSH2 domain in PLC-γ1. This conformation change results in the interaction between PLC-γ1 SH3 domain and Akt proline motifs. This model may also apply to the interaction between PLC-γ1 SH3 domain and other proteins, such as PLD_2 (Jang et al., 2003), PIKE (Ye et al., 2002), dynamin (Choi et al., 2004), and Emt (Perez-Villar and Kanner, 1999). Furthermore, the interaction between PLC-γ1 and Akt causes phosphorylation of PLC-γ1 S1248 and plays important role in EGF-stimulated cell motility.

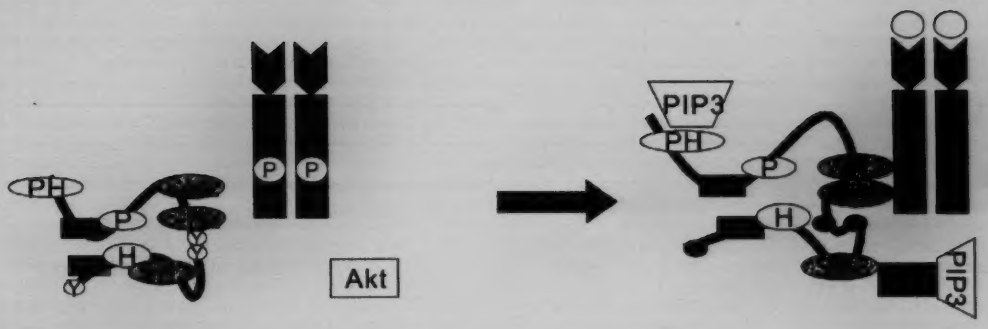

Figure 7.3 Model to illustrate EGF-induced interaction between Akt and PLC-γ1. Before EGF stimulation, both PLC-γ1 and Akt are located in the cytosol. The conformation of inactive PLC-γ1 blocks the interaction between PLC-γ1 the SH3 domain and the proline-rich motifs of Akt (left). After EGF stimulation, PLC-γ1 translocated to the plasma membrane, and its Y783 was phosphorylated by EGFR, which exposes its SH3 domain for interaction. Simultaneously, Akt was activated by EGFR through PI3K and translocated to the plasma membrane. At the plasma membrane, the PLC-γ1SH3 domain and Akt proline-rich motifs bind to each other (right).

7.8 Future Directions

Although our model depicts a detailed mechanism for the spatial regulation of RTKs and the regulation by PLC-γ1, there are still some issues in the model that require testing, and some questions that need to be answered by future studies. Firstly, my thesis work provides strong support for the generation of specific signals from endosomes that are sufficient to cause biological effects, such as mitogenesis and anti-apoptosis. Our results also suggest that endosomal signaling has identical effects on both PDGFR and EGFR systems. But in other RTK systems, such as TrkA and Insulin Growth Factors Receptor, the physiological role of endosomal signaling has not been clearly identified. The use of our systems which can specifically activate RTKs in endosomes will also provide the direct support for or against endosomal signaling in these cases as well. Moreover, it also can determine whether endosomal signaling has some specific roles, such as promoting neuronal differentiation by endosomal signaling of TrkA.

Secondly, the EGFR system appears to have the greatest versatility. Some ligands, such as TGF-α, rapidly dissociate and are degraded within endosomes, while others, such as EGF, remain associated and are resistant to endosomal degradation. This ligand-specific processing and the consequent receptor trafficking may play an important role in generating a variety of biological responses. The next step that needs to be taken is to understand the mechanistic basis of selective endosomal signaling and its biological consequences. What is the relative contribution of signal duration and specific substrate assembly? What determines where a RTK can productively interact with its signaling

partners? Answers to these questions will require methods to separate and analyze the composition of endosome signaling complexes. Also required is a better understanding of the link between substrate phosphorylation, induction kinetics, and biological responses.

Thirdly, our results suggest that Akt regulates PLC-γ1 phosphorylation and is involved in cell motility. How PLC-γ1 is involved in the cell motility, however, remains unknown. It was reported that inhibitors of PKC which inhibit serine phosphorylation of PLC-γ1 also inhibit the tyrosine phosphorylation and catalytic activity PLC-γ1 (Yamada *et al.*, 1992). Wortmannin, an inhibitor of PI3K, which inhibits Akt indirectly, also inhibits the catalytic activity of PLC-γ1. PLC-γ1 catalytic activity is involved in EGF mediated cell motility (Chen *et al.*, 1994). Therefore, Akt may regulate EGF mediated cell motility through regulating the catalytic activity of PLC-γ1.

However, other mechanisms may also exist. Recently, the SH3 domain of PLC-γ1 was suggested to serve as a GEF for PIKE and dynamin. PLC-γ1 was reported to associate with Cdc42 in an EGF-dependent manner (Chou et al., 2003), and leukotriene D4 induced association of active RhoA with PLC-γ1 in intestinal epithelial cells (Thodeti et al., 2002). Since Rho GTPases are always considered as important regulators of cell motility, PLC-γ1 may regulate cell motility through Rho GTPases. We can check whether PLC-γ1 can regulate the activity of Rho GTPases and whether this regulation is through the direct association between them or through the indirect association mediated by some adaptor proteins.

Lastly, whether the interaction between PLC-γ1 and Akt will also modulate Akt

activity and function is still not clear. It was reported that PLC-γ2, a homologue of PLC-γ1, is required for B cell receptor-mediated cell survival (Bell *et al.*, 2004). Akt activation is abolished by the PLC-γ1 inhibitor U73122 which suggests a possible role of PLC-γ1 in EGF-induced cell survival through the interaction with Akt (Deb *et al.*, 2004). Therefore, whether the interaction between PLC-γ1 and Akt regulates cell survival warrants future research.

The findings from this dissertation have further advanced our knowledge regarding the regulation of RTK signaling by endocytosis and by PLC-γ1. My data suggests that receptor trafficking and signaling are tightly coupled together. The model generated from my study is another step towards constructing a more complete and detailed model regarding the regulation of RTK signaling. These data will certainly help us to understand how the uncontrolled RTK signaling contributes to cancer development and to provide a way to intervene when this uncontrolled signaling cause human cancers.

Chapter 8

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